

**UNIVERSITY OF EDINBURGH**

**Vascular Regulation of  
Endogenous Fibrinolysis in Man**

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## **CONTENTS**

**ABSTRACT (REGULATION 1.1.3)**

**STATEMENT (REGULATION 1.1.4)**

**ACKNOWLEDGEMENTS**

**PUBLICATIONS**

**SECTION 1 CARDIOVASCULAR PHYSIOLOGY (Publications 1-10)**

**SECTION 2 CARDIOVASCULAR RISK FACTORS (Publications 11-16)**

**SECTION 3 ISCHAEMIC HEART DISEASE (Publications 17-25)**

**SECTION 4 HEART FAILURE (Publications 26-30)**

**SECTION 5 MISCELLANEOUS TOPICS**

**AND REVIEWS (Publications 31-37)**



## ABSTRACT (REGULATION 1.1.3)

The initiation, modulation and resolution of thrombus associated with eroded or unstable coronary plaques are critical determinants of acute coronary events. This itself is dependent on the cellular function of the surrounding endothelium and vascular wall. In particular, the regulation of vascular tone and the acute release of tissue plasminogen activator by the endothelium make important contributions to the defence against intravascular thrombosis. These aspects of endothelial function will provide major new insights into the pathophysiology of cardiovascular disease, and to shape future therapeutic interventions.

The experimental medicine models presented here originate from an early interest in exploring these novel aspects of endothelial function, and in particular, the acute release of the endothelium-derived fibrinolytic factor, tissue plasminogen activator. This model was initiated, developed and applied to a diverse range of physiological and pathophysiological circumstances that have informed health and disease. Publications are grouped into 5 main sections.

**Section 1** describes the early validation and exploration of the model to stimulate acute tissue plasminogen activator release using a variety of physiological and pathophysiological mediators. This describes the early identification of potent stimulators of acute tissue plasminogen activator release including substance P, bradykinin, desmopressin and thrombin receptor agonists. This work was extended to demonstrate the important role of nitric oxide and vascular inflammation in this process. Subsequent sections cover the application of these techniques in patient populations with a predisposition to cardiovascular disease (**Section 2**) as well as with established (**Section 3**) and end-stage disease (**Section 4**). These sections also explore the potentially important modulation of the renin-angiotensin system with particular reference to angiotensin-converting enzyme inhibition and the role of bradykinin. **Section 5** contains some miscellaneous work and reviews of the subject area bring together the key aspects of the field as well as an important study to address the vascular endothelial function in patients with a rare haematological deficiency, type 3 von Willebrand disease.

## **STATEMENT (REGULATION 1.1.4)**

(a) Part of this work has been previously presented in my PhD thesis (University of Edinburgh 2000). Some of this work has previously been presented, or is under submission, in MD and PhD theses by Drs Chia, Cruden, Mills, Robinson, and Witherow (all submitted to the University of Edinburgh). This Doctorate of Science thesis has not been previously presented for this degree elsewhere. All sources of information have been acknowledged.

(b) This thesis represents research undertaken whilst at the University of Edinburgh over the last 14 years. The substantial body of work described is my own. I have been fortunate in gaining the advice and assistance of many colleagues, and much of the work has been conducted in collaboration with young enthusiastic clinicians who have conducted their postgraduate education under my supervision. In particular, I was responsible for the initial conception, design, methodology development, study conduct, validation, and securing of funding. The work has been presented and published by myself or with the assistance and under the close supervision of junior investigators.

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## **SECTION 1**

### **CARDIOVASCULAR PHYSIOLOGY (Publications 1-10)**

## **PUBLICATION 1**

# An *in vivo* Model for the Assessment of Acute Fibrinolytic Capacity of the Endothelium

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## Summary

The effects on blood flow and plasma fibrinolytic and coagulation parameters of intraarterial substance P, an endothelium dependent vasodilator, and sodium nitroprusside, a control endothelium independent vasodilator, were studied in the human forearm circulation. At sub-systemic locally active doses, both substance P (2-8 pmol/min) and sodium nitroprusside (2-8 µg/min) caused dose dependent vasodilatation ( $p < 0.001$  for both) without affecting plasma concentrations of PAI-1, von Willebrand factor antigen or factor VIII:C activity. Substance P caused local increases in t-PA antigen and activity ( $p < 0.001$ ) in the infused arm while sodium nitroprusside did not. At higher doses, substance P increased blood flow and t-PA concentrations in the noninfused arm. We conclude that brief, locally active and sub-systemic infusions of intraarterial substance P cause a rapid and substantial local release of t-PA which appear to act via a flow and nitric oxide independent mechanism. This model should provide a useful and selective method of assessing the *in vivo* capacity of the forearm endothelium to release t-PA acutely.

## Introduction

Endothelial cells of the precapillary arterioles and postcapillary venules (1) synthesise and constitutively secrete tissue type plasminogen activator (t-PA) and its inhibitor, plasminogen activator inhibitor type 1 (PAI 1). The release of t-PA may be rapidly increased through the translocation of a dynamic intracellular storage pool (2) in response to stimulation by blood coagulation and humoral factors (3). Acute t-PA release plays a pivotal role in endogenous fibrinolysis and this is exemplified by a plasminogen activator deficient gene knockout mouse model that exhibits an increased incidence of endotoxin-induced thrombosis (4). The time course of t-PA release is important, with thrombus dissolution being much more effective if t-PA is incorporated during, rather than after, thrombus formation (5, 6). Thus, the speed with which, and extent to which, t-PA can be released from endothelial cells may have a substantial impact on the efficacy of endogenous fibrinolysis.

When studying *in vivo* vascular responses in man, systemic drug administration can cause concomitant effects on other organ systems, such as the liver, brain, kidney and heart, as well as influence neuro-humoral reflexes through changes in systemic haemodynamics. There-

fore, because of these confounding influences, vascular and humoral responses cannot be wholly attributed to a direct effect of the drug on the blood vessels. Endogenous fibrinolysis in man has been assessed using systemic infusion of agents such as desmopressin (7, 8) and angiotensin II (9). These agents are vasoactive, producing changes in blood pressure and regional blood flow, as well as having widespread effects on many tissues. Thus, changes in systemic fibrinolytic parameters might be attributable to a number of factors including changes in hepatic release and clearance of t-PA and PAI 1, and the concomitant release of other stimulatory, vasoactive and humoral mediators. In contrast, the use of bilateral forearm blood flow measurements coupled with unilateral brachial artery infusion of vasoactive drugs at sub-systemic, locally active doses, provides a powerful and reproducible method of directly assessing vascular responses *in vivo* (10, 11). Combined with bilateral forearm venous sampling, this technique permits the assessment of local release of tissue and endothelium derived factors (12).

Substance P is a member of the tachykinin family of peptides, acting through stimulation of neurokinin receptors, and having a particularly high affinity for the type 1 (NK<sub>1</sub>) receptor (13). It is widely distributed in the body and has actions as a central, peripheral and enteric neurotransmitter (14-17), inflammatory mediator (18-20) and neurohumoral regulator (15, 21, 22). When given intra-arterially in man, substance P enhances local fibrinolytic activity through an unknown mechanism (23) and has actions as a potent vasodilator (24-26) through an endothelium dependent (27) and predominantly nitric oxide mediated mechanism (28, 29). The determination of vascular responses to the intraarterial infusion of endothelial cell stimulants such as substance P has been widely used to assess the integrity of endothelium dependent vasodilatation in health and disease (30-33).

The initial aim of the present study was to assess the acute release of coagulation and fibrinolytic factors within the forearm vascular bed in response to intraarterial substance P using an ascending dose design to define the dose at which systemic effects intervene. Thereafter, endothelial cell release of these factors was assessed in response to locally active doses of substance P and a control endothelium-independent nitric oxide donor, sodium nitroprusside (32, 33).

## Materials and Methods

### Subjects

Sixteen healthy non smoking men aged between 20 and 34 years participated in two studies which were undertaken with the approval of the local research ethics committee and in accordance with the Declaration of Helsinki. The written informed consent of each subject was obtained before entry into the study. None of the subjects received vasoactive or nonsteroidal anti-inflammatory drugs in the week before each phase of the study, and all abstained from

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alcohol for 24 h, and from food and caffeine-containing drinks for at least 5 h, before each study. All studies were performed in a quiet, temperature controlled room maintained at 23.5–24.5°C.

#### Intraarterial Administration

The brachial artery of the nondominant arm was cannulated with a 27-standard wire gauge steel needle (Cooper's Needle Works Ltd, Birmingham, UK) under 1% lignocaine (Xylocaine; Astra Pharmaceuticals Ltd, Kings Langley, UK) local anaesthesia and attached to a 16-gauge epidural catheter (Portex Ltd, Hythe, UK). Patency was maintained by infusion of saline via an IVAC P1000 syringe pump (IVAC Ltd, Basingstoke, UK). The total rate of intra-arterial infusions was maintained constant throughout all studies at 1 ml/min.

#### Drugs

Pharmaceutical-grade substance P (Clinalfa AG, Läufelfingen, Switzerland) and sodium nitroprusside (Nipride; Roche, Welwyn Garden City, UK) were administered following dissolution in saline (0.9%; Baxter Healthcare Ltd, Thetford, UK).

#### Forearm Blood Flow and Blood Pressure

Blood flow was measured in both forearms by venous occlusion plethysmography using mercury-in-silastic strain gauges applied to the widest part of the forearm (10, 11). During measurement periods the hands were excluded from the circulation by rapid inflation of the wrist cuffs to a pressure of 220 mmHg using E20 Rapid Cuff Inflators (D.E. Hokanson Inc, Washington, USA). Upper arm cuffs were inflated intermittently to 40 mmHg for 10 s in every 15 s to achieve venous occlusion and obtain plethysmographic recordings. Analogue voltage output from an EC-4 Strain Gauge Plethysmograph (D.E. Hokanson) was processed by a MacLab<sup>®</sup> analogue-to-digital converter and Chart v3.3.8 software (AD Instruments Ltd, Castle Hill, Australia) and recorded onto a MacIntosh Classic II computer (Apple Computers Inc, Cupertino, USA). Calibration was achieved using the internal standard of the plethysmograph.

**Table 1** Dose ranging study: systemic haemodynamics, forearm blood flow, estimated net t-PA antigen and activity release, and vWf concentrations and factor VIII:C activity in the infused and non-infused forearms at baseline and during substance P infusion (n = 7). \* p = 0.05 (t-test); ‡ p < 0.001 (ANOVA); § p = 0.06 (ANOVA)

		Substance P Infusion (pmol/min)			
		Baseline	2	16	64
<b>Blood Pressure (mmHg)</b>	<b>Systolic</b>	140 ± 6	139 ± 4	139 ± 4	138 ± 7
	<b>Diastolic</b>	70 ± 4	68 ± 5	69 ± 6	65 ± 4
<b>Heart Rate (/min)</b>		57 ± 4	56 ± 4	61 ± 3	63 ± 3
<b>Percentage Change in Forearm Blood Flow</b>		-	233 ± 59 %	466 ± 197 %	221 ± 105 %‡
<b>Absolute Forearm Blood Flow (ml/100 ml/min)</b>	<b>Non-infused Arm</b>	3.4 ± 0.4	3.6 ± 0.5	4.6 ± 0.9*	8.8 ± 1.7‡
	<b>Infused Arm</b>	3.7 ± 0.7	11.7 ± 1.5	20.9 ± 2.8	21.5 ± 2.4‡
<b>Estimated Net Release</b>	<b>t-PA Antigen (ng/100ml/min)</b>	0.7 ± 0.8	3.8 ± 3.1	18.2 ± 5.3	78.4 ± 25.3‡
	<b>t-PA Activity (IU/100ml/min)</b>	-0.1 ± 0.1	0.9 ± 0.6	20.0 ± 7.8	45.6 ± 9.6‡
<b>von Willebrand Factor (IU/ml)</b>	<b>Non-infused Arm</b>	0.72 ± 0.06	0.84 ± 0.07	0.99 ± 0.04	0.89 ± 0.04§
	<b>Infused Arm</b>	0.81 ± 0.07	0.93 ± 0.09	1.04 ± 0.15	1.02 ± 0.15
<b>Factor VIII:C (IU/ml)</b>	<b>Non-infused Arm</b>	0.49 ± 0.05	0.56 ± 0.05	0.58 ± 0.05	0.53 ± 0.05
	<b>Infused Arm</b>	0.52 ± 0.03	0.57 ± 0.04	0.56 ± 0.05	0.49 ± 0.03

Blood pressure was monitored in the noninfused arm at intervals throughout each study using a semiautomated noninvasive oscillometric sphygmomanometer (34) (Takeda UA 751, Takeda Medical Inc, Tokyo, Japan).

#### Venous Sampling and Assays

Venous cannulae (19G) were inserted into large subcutaneous veins of the antecubital fossa in both arms as described previously (12). Ten ml of blood was withdrawn simultaneously from each arm and collected into acidified buffered citrate (Biopool<sup>®</sup> Stabilyte™, Umeå, Sweden) and citrate (Monovette<sup>®</sup>, Sarstedt, Nümbrecht, Germany) tubes, and kept on ice before being centrifuged at 2,000 g for 30 min at 4°C. Platelet free plasma was decanted and stored at -80°C before assay.

Plasma PAI-1 and t-PA antigen concentrations were determined using an enzyme-linked immunosorbent assay; Coaliza<sup>®</sup> PAI-1 (35) and Coaliza<sup>®</sup> t-PA (36) (Chromogenix AB, Mölndal, Sweden) respectively. Plasma PAI-1 and t-PA activities were determined by a photometric method, Coatest<sup>®</sup> PAI-1 (37) and Coaset<sup>®</sup> t-PA (38) (Chromogenix AB). Intraassay coefficients of variation were 7.0% and 5.5% for t-PA and PAI-1 antigen, and 4.0% and 2.4% for activity, respectively. Interassay coefficients of variability were 4.0%, 7.3%, 4.0% and 7.6% respectively. The sensitivities of the assays were 2.5 ng/ml, 0.5 ng/ml, 5 AU/ml and 0.10 IU/ml respectively. Von Willebrand factor (vWf) antigen was determined (39) using an enzyme-linked immunosorbent assay (Dako A/S, Glostrup, Denmark) with a sensitivity of 0.05 IU/ml. The intra-assay and interassay coefficients of variability were 5.2% and 7.3% respectively. Factor VIII:C procoagulant activity was determined using a standard one stage assay on an ACL-3000+ coagulometer (Instrumentation Laboratory, Warrington, UK).

#### Study Design

Subjects rested recumbent throughout each study. Strain gauges and cuffs were applied and the brachial artery of the nondominant arm cannulated. Measurements of forearm blood flow were made between 3 and 6 min of each infusion period unless otherwise stated. Before participating in one of the following protocols, saline was infused for the first 30 min to allow time for

equilibration, with forearm blood flow measured every 10 min and the final measurement taken as basal blood flow.

#### Dose Ranging Study

In seven men, intrabrachial substance P was administered in incremental doubling doses from 0.5 to a maximum of 128 pmol/min for 6 min at each dose and was followed by 30 min saline infusion. Venous samples were taken at baseline, following 2 pmol/min, 16 pmol/min and the maximal dose of substance P, and after the final 30 min saline infusion.

#### Local Forearm Study

Twelve men were given intraarterial doubling doses of substance P at 2, 4 and 8 pmol/min for 10 min at each dose, and sodium nitroprusside at 2, 4 and 8 µg/min for 10 min at each dose, separated by a 30 min saline infusion. Substance P and sodium nitroprusside were given single blind, in randomised order. Venous samples were obtained at the end of each period of saline infusion and with each dose of substance P and sodium nitroprusside.

#### Data Analysis and Statistics

Plethysmographic data were extracted from the Chart data files and forearm blood flows were calculated for individual venous occlusion cuff inflations by use of a template spreadsheet (Excel v4.0; Microsoft Corporation, Cambridge, USA). Recordings from the first 60 s after wrist cuff inflation were not used because of the reflex vasoconstriction this causes (10, 11). Usually, the last five flow recordings in each 3 min measurement period were calculated and averaged for each arm. To reduce the variability of blood flow data, the ratio of flows in the two arms was calculated for each time point; in effect using the non-infused arm as a contemporaneous control for the infused arm (10, 11).

Estimated net release of t-PA activity and antigen was defined as the product of the infused forearm plasma flow (based on the hematocrit, Hct and the infused forearm blood flow, FBF) and the concentration difference between the infused ( $[t-PA]_{Inf}$ ) and non-infused arms ( $[t-PA]_{Noninf}$ ).

$$\text{Estimated net t-PA release} = \text{FBF} \times [1 - \text{Hct}] \times ([t-PA]_{Inf} - [t-PA]_{Noninf})$$

Data were examined, where appropriate, by two way analysis of variance (ANOVA) with repeated measures and two tailed paired Student's *t*-test using Excel v4.0 (Microsoft). All results are expressed as means ± standard errors of the mean. Statistical significance was taken at the 5% level.

#### Results

All subjects were normotensive (Tables 1 and 2) and had a normal fasting lipid profile with a mean total cholesterol concentration of  $3.90 \pm 0.16$  mM ( $150 \pm 6$  mg/dl), high density lipoprotein cholesterol concentration of  $1.02 \pm 0.05$  mM ( $39 \pm 2$  mg/dl) and triglyceride concentration of  $0.87 \pm 0.08$  mM ( $77 \pm 7$  mg/dl).

#### Dose Ranging Study

There were no significant changes in arterial pressure or heart rate throughout the study. Substance P caused an increase in blood flow of the infused forearm ( $p < 0.001$ ) from a baseline of  $3.7 \pm 0.7$  ml/100ml/min to a maximum of  $22 \pm 2.4$  ml/100ml/min at 64 pmol/min in a dose-dependent manner (Fig. 1). Five subjects received 64 pmol/min and 2 received 128 pmol/min as the maximum dose, further infusion of substance P being discontinued because of forearm skin oedema and facial flushing. There was a significant

Table 2 Local forearm study: systemic haemodynamics, forearm blood flow, estimated net t-PA antigen and activity release, and vWf concentrations and factor VIII : C activity in the infused and non-infused forearms at baseline and during sodium nitroprusside and substance P infusion (n = 12). \*  $p < 0.001$  (ANOVA)

		Sodium Nitroprusside (µg/min)				Substance P (pmol/min)			
		0	2	4	8	0	2	4	8
Blood Pressure (mmHg)	Systolic	132 ± 4	133 ± 4	135 ± 4	133 ± 4	131 ± 3	138 ± 4	135 ± 3	136 ± 3
	Diastolic	70 ± 2	70 ± 2	69 ± 2	68 ± 1	70 ± 2	71 ± 3	70 ± 2	68 ± 3
Heart Rate (/min)		63 ± 2	61 ± 2	61 ± 2	61 ± 2	64 ± 2	61 ± 2	61 ± 2	60 ± 2
Percentage Change in Forearm Blood Flow		-	247 ± 53	370 ± 73	541 ± 111 *	-	193 ± 41	286 ± 77	383 ± 79
Absolute Forearm Blood Flow (ml/100 ml/min)	Non-infused Arm	4.1 ± 0.5	4.1 ± 0.6	3.8 ± 0.6	3.8 ± 0.6	3.8 ± 0.6	4.0 ± 0.6	4.0 ± 0.6	3.9 ± 0.7
	Infused Arm	4.6 ± 0.7	11.6 ± 0.8	14.8 ± 1.1	18.1 ± 1.4 *	4.4 ± 0.6	11.1 ± 1.2	13.5 ± 1.4	16.5 ± 1.7
Estimated Net Release	t-PA Antigen (ng/100ml/min)	-0.5 ± 0.3	-1.6 ± 0.8	-0.5 ± 1.4	0.5 ± 2.7	0.0 ± 0.2	2.9 ± 1.3	7.3 ± 2.1	15.6 ± 3.7
	t-PA Activity (IU/100ml/min)	-0.2 ± 0.1	-0.8 ± 1.0	-0.7 ± 1.7	-1.1 ± 3.5	0.3 ± 0.2	5.0 ± 1.7	8.8 ± 2.5	17.8 ± 3.9
von Willebrand Factor (IU/ml)	Non-infused Arm	0.66 ± 0.08	0.60 ± 0.07	0.64 ± 0.10	0.68 ± 0.08	0.65 ± 0.10	0.59 ± 0.08	0.61 ± 0.10	0.60 ± 0.13
	Infused Arm	0.64 ± 0.08	0.62 ± 0.09	0.65 ± 0.09	0.67 ± 0.09	0.62 ± 0.07	0.58 ± 0.09	0.58 ± 0.10	0.63 ± 0.08
Factor VIII:C (IU/ml)	Non-infused Arm	0.61 ± 0.08	0.57 ± 0.08	0.59 ± 0.08	0.62 ± 0.08	0.57 ± 0.09	0.60 ± 0.08	0.59 ± 0.09	0.60 ± 0.09
	Infused Arm	0.64 ± 0.07	0.64 ± 0.09	0.65 ± 0.09	0.67 ± 0.09	0.53 ± 0.08	0.57 ± 0.10	0.57 ± 0.10	0.63 ± 0.09



increase in the blood flow of the contralateral, noninfused arm ( $p = 0.001$ , ANOVA) which was apparent from 16 pmol/min ( $p = 0.05$ ). The relative percentage increase in blood flow of the infused compared with the noninfused arm was dose-dependent, peaking at 32 pmol/min before declining at 64 pmol/min (Fig. 1).

Substance P caused increases in plasma t-PA antigen and activity concentrations in the infused ( $p < 0.001$  for both) and noninfused arm ( $p \leq 0.003$  for both) which were dose-dependent (Fig. 2). Plasma from the infused arm demonstrated significantly greater increases in both t-PA activity and antigen concentrations than the noninfused arm ( $p < 0.001$ ). At the maximal dose, mean t-PA activity increased by 630% in the infused arm and 210% in the noninfused arm, whilst mean t-PA antigen increased by 240% and 62% respectively.

There were no significant or consistent changes in plasma PAI-1 antigen or activity concentrations in the infused arm. There was a significant decrease in the plasma PAI-1 activity in the noninfused arm ( $p = 0.03$ ) although PAI-1 antigen concentrations did not change significantly ( $p = 0.64$ ). There were no significant changes in plasma vWf concentration or factor VIII:C activity in either arm (Table 1).

### Local Forearm Study

There were no significant changes in blood pressure, heart rate or forearm blood flow in the contralateral arm throughout the study (Table 2).

Both substance P and sodium nitroprusside caused selective increases in forearm blood flow in the infused arm ( $p < 0.001$  for both) in a dose dependent manner (Table 2). Substance P caused a selective and dose dependent increase in the estimated net release ( $p < 0.001$  for both) and venous plasma concentrations ( $p < 0.001$  for both) of both t-PA activity and antigen (Table 2; Fig. 3). In contrast, there were no significant changes in plasma t-PA activity or antigen concentrations in the noninfused arm, or in PAI-1 antigen and activity, vWf or factor VIII:C concentrations in either arm (Table 2; Fig. 3). There were no significant changes in t-PA, PAI-1, vWf or factor VIII:C in either arm during sodium nitroprusside infusion (Table 2; Fig. 3).

### Discussion

We have shown, for the first time, that intraarterial substance P administration causes acute, selective and substantial t-PA release *in vivo* in man. At both systemic and locally active doses, substance P

causes t-PA release from the forearm vascular bed without significant effects on the release of PAI-1, vWf and factor VIII:C. This model provides a selective *in vivo* method of assessing acute t-PA release from the endothelium in man.

Intrabrachial substance P has been shown previously to induce local fibrinolysis in the forearm (23) although the mechanism of this effect had not been determined. However, taken together with our findings, it is apparent that this enhancement of fibrinolytic activity is, at least in part, mediated through t-PA release. Previously, bradykinin was thought to be one of the most potent agents causing t-PA release in animals (3, 40) and man (3, 41). However, a recent study in man (41) using systemic intravenous bradykinin administration at doses of up to 380 pmol/kg/min, did not show a significant release of t-PA antigen except in the presence of angiotensin converting enzyme inhibition and alterations in systemic haemodynamic parameters. Jern and colleagues (42) have shown a significant net local release of t-PA antigen and activity in response to intra-brachial methacholine. However, a significant increase in venous concentrations of t-PA antigen was not detected and, although measurement of arteriovenous differences should enhance the accuracy of assessing local tissue release, there were no significant increases in the arteriovenous gradients of t-PA antigen or activity. Indeed, the clearest changes were observed in the arterial t-PA activity which should have remained constant, suggesting that there was systemic stimulation of t-PA release in this study.

Venous plasma t-PA concentrations obtained from a given tissue bed are composed of three components; circulating arterial t-PA, basal or constitutive endothelial cell release of t-PA and facultative or stimulated endothelial cell release of t-PA. The net tissue release of t-PA is equivalent to the product of the plasma flow through the tissue and the arteriovenous difference in plasma t-PA concentrations across it. In the absence of endothelial cell stimulation, but with an increase in blood flow across the tissue bed, venous plasma t-PA concentrations would be expected to fall secondary to a dilutional effect. However, this ignores the potential for clearance of t-PA across the vascular bed (43), and stimulation of its release by shear stress and flow (44, 45). Without measuring the arteriovenous concentration gradient across the forearm, net tissue release can only be derived and estimated. However, arterial sampling requires the insertion of large bore cannulae which do not lend themselves to multiple cannulations within the same subject. There is also the potential to introduce artefact from the presence of a larger thrombogenic surface given that activated factor Xa is the most

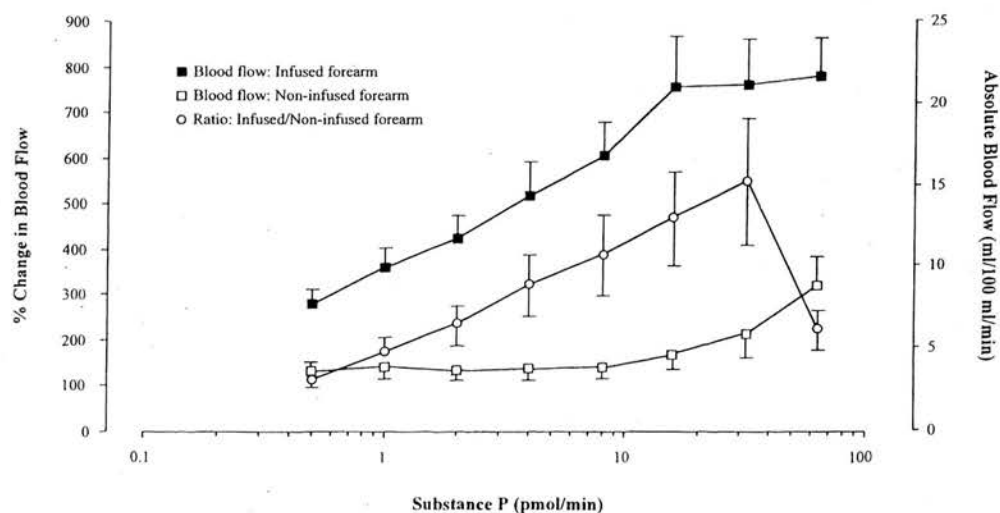


Fig. 1 Dose ranging study: percentage change and absolute forearm blood flow responses to incremental doses of substance P ( $n = 7$ ).

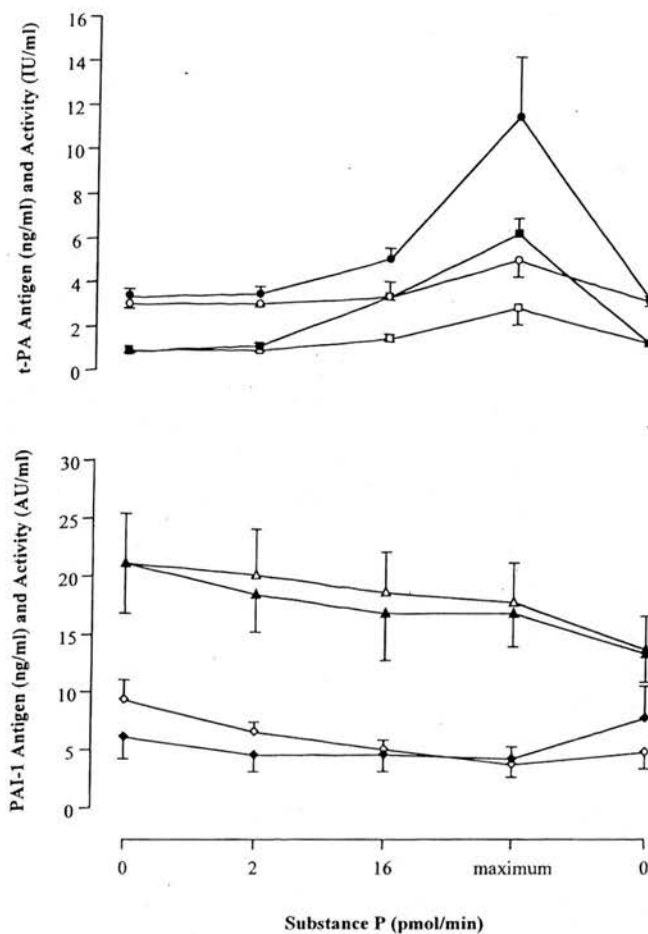


Fig. 2 Dose ranging study: venous plasma tissue plasminogen activator (t-PA) antigen (○) and activity (□), and plasminogen activator inhibitor type 1 (PAI-1) antigen (Δ) and activity (◇) concentrations in the infused (closed symbols) and non-infused (open symbols) forearms in response to substance P infusions (n = 7). Maximum substance P dose was 64 pmol/min in 5 subjects and 128 pmol/min in 2 subjects.  $p < 0.001$  for all t-PA concentrations (ANOVA)

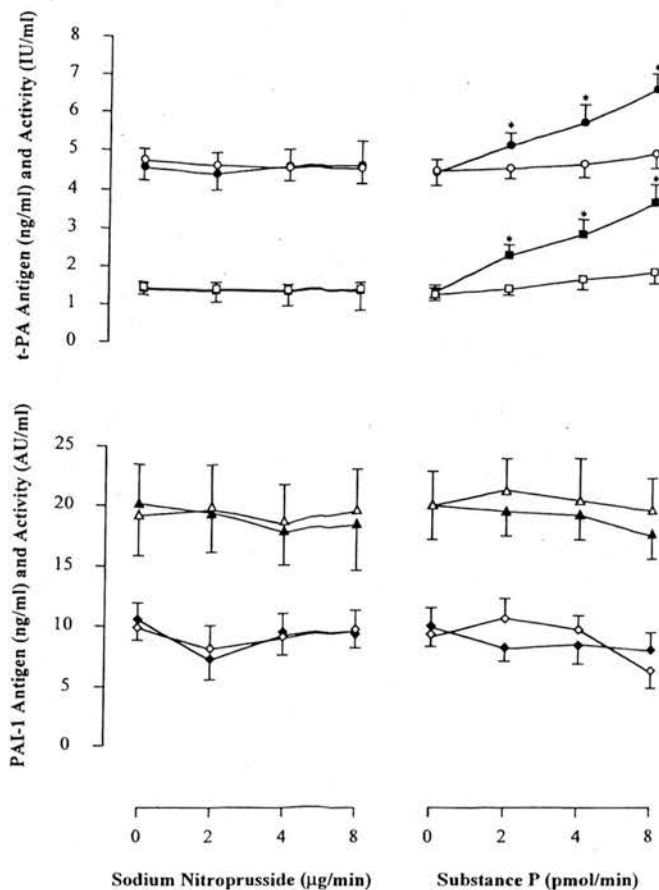


Fig. 3 Local forearm study: venous plasma tissue plasminogen activator (t-PA) antigen (○) and activity (□), and plasminogen activator inhibitor type 1 (PAI-1) antigen (Δ) and activity (◇) concentrations in the infused (closed symbols) and non-infused (open symbols) forearms in response to sodium nitroprusside and substance P infusions (n = 12). \*  $p < 0.001$  (ANOVA)

potent stimulant for t-PA release yet known (3). Rather than assessing arteriovenous differences, we have compared venous plasma t-PA concentrations between infused and non-infused arms and have used very fine gauge arterial cannulae for drug administration only. This method may potentially underestimate the net release of t-PA and fail to detect a modest effect due to the potential flow dependent, dilutional changes in venous concentrations. However, typical resting arteriovenous differences are only ~10% of the total venous concentration (42, 46) and the basal constitutive release of t-PA antigen is ~0.9 ng/100 ml of tissue/min in the forearm (42). Thus, in the presence of large increases in t-PA release, the dilutional effect of increased blood flow on constitutive t-PA release will be reduced. Indeed, using this bilateral venous sampling methodology, we have been able to demonstrate a substantial, dose-dependent release of t-PA from the forearm vascular bed in response to substance P infusion. Moreover, despite *in vitro* evidence that t-PA release may be influenced by shear stress (44, 45), we have found that the endothelium independent nitric oxide donor, sodium nitroprusside, has no significant effect on the venous t-PA concentrations despite comparable increases in blood flow to those with substance P. Sodium nitroprusside is known to have no direct effect on the endothelial cell release of t-PA and PAI-1 *in vitro* (47) and, therefore, it is likely that either shear stress and flow depen-

dent stimulation of endothelial cell t-PA release is counterbalanced by the potential dilutional effects of increased flow, or that this theoretical flow dependence of venous concentrations is negligible. This also indicates that increases in nitric oxide and blood flow are not sufficient in themselves to release t-PA from the endothelium. However, it remains a possibility that the L-arginine : nitric oxide pathway plays a role in substance P-induced t-PA release and requires further studies using a combined infusion of substance P and a nitric oxide synthase inhibitor such as L-N<sup>G</sup>-monomethyl arginine.

Although we have produced substantial increases in both t-PA activity and antigen, we did not detect release of PAI-1, or the coagulation factors, vWf and factor VIII : C. This would indicate that these agents are not stored in a rapidly translocatable pool within the endothelial cells of the forearm vascular bed or that they are not released in response to substance P over the time course and at the doses used here. However, protracted endothelial cell stimulation may release these factors (7, 9). In this respect, it is interesting to note the time dependent reduction in PAI-1 concentrations seen in our studies, although these only achieved statistical significance in the non-infused arm during the dose ranging study. The most likely explanation for a reduction in PAI-1 is that the released and active t-PA is complexed by circulating PAI-1 and subsequently cleared from the circulation by the liver (48).



Thus, substantial local t-PA release will tend to reduce systemic PAI-1 concentrations in the short term, as seen with the administration of pharmacological doses of t-PA (49).

As anticipated, local substance P infusion did not affect the rate of release of hepatically derived factor VIII : C. In contrast, it is perhaps surprising that we did not observe a rise in plasma vWf concentrations to accompany the release of t-PA. To date, stimulation of t-PA release using a wide range of secretagogues such as thrombin, vasopressin, bradykinin, histamine and desmopressin, has invariably been accompanied by concomitant vWf release (7, 50, 51). However, we were unable to detect an acute local release of vWf even in the presence of high local concentrations of substance P in the dose ranging study. This novel selectivity suggests that the endothelium is able to mobilise different cytoplasmic storage pools in response to specific (NK<sub>1</sub>) receptor stimulation. Further studies with more prolonged infusions of substance P would be required to determine whether the endothelial cell vWf or PAI-1 release is delayed or is truly not influenced by substance P.

In summary, brief, locally active and subsystemic infusions of intra-arterial substance P produce a rapid and substantial increase in plasma t-PA activity and antigen concentrations across the forearm bed which appear to act via a flow and nitric oxide independent mechanism. This model provides a powerful method of assessing the *in vivo* capacity of the endothelium to acutely release t-PA within the forearm vascular bed and would be applicable to the assessment of diseases associated with endothelial dysfunction, such as hypercholesterolemia (52).

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## **PUBLICATION 2**

## The L-arginine/nitric oxide pathway contributes to the acute release of tissue plasminogen activator in vivo in man

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### Abstract

**Objective:** Effective endogenous fibrinolysis requires rapid release of endothelial tissue plasminogen activator (t-PA). Using the nitric oxide synthase inhibitor, L-N<sup>G</sup>-monomethylarginine (L-NMMA), we examined the contribution of endogenous nitric oxide to substance P-induced t-PA release in vivo in man. **Methods:** Blood flow and plasma fibrinolytic and haemostatic factors were measured in both forearms of 8 healthy male volunteers who received unilateral brachial artery infusions of substance P (2–8 pmol/min) and L-NMMA (1–4 µg/min). **Results:** Substance P caused dose-dependent increases in blood flow ( $P < 0.001$ ) and plasma t-PA antigen ( $P = 0.04$ ) and activity ( $P < 0.001$ ) concentrations confined to the infused forearm, but had no effect on plasminogen activator inhibitor type 1 (PAI-1) or von Willebrand factor concentrations. In the presence of L-NMMA, substance P again caused significant increases in blood flow ( $P < 0.001$ ) and t-PA antigen ( $P = 0.003$ ) and activity ( $P < 0.001$ ) concentrations but these increases were significantly less than with substance P alone ( $P < 0.001$ ,  $P = 0.05$  and  $P < 0.01$ , respectively). L-NMMA alone significantly reduced blood flow in the infused arm, but had no measurable effect on t-PA or PAI-1 concentrations. **Conclusions:** The L-arginine/nitric oxide pathway contributes to substance P-induced t-PA release in vivo in man. This provides an important potential mechanism whereby endothelial dysfunction increases the risk of atherothrombosis through a reduction in the acute fibrinolytic capacity. © 1998 Elsevier Science B.V. All rights reserved.

**Keywords:** Thrombolysis; Endothelial factor; Nitric oxide; Blood flow; Endothelial function

### 1. Introduction

The endogenous fibrinolytic system can have important clinical effects as exemplified by the observation that in ~30% of patients with an acute myocardial infarction, the infarct-related artery spontaneously reperfuses within 12 h [1–3]. The ability of the endothelium to release tissue plasminogen activator (t-PA) rapidly is crucial if endogenous fibrinolysis within the arterial circulation is to be effective, with thrombus dissolution being much more effective if t-PA is incorporated during, rather than after, thrombus formation [4,5]. Epidemiological studies in a

healthy male population and patients with ischaemic heart disease have shown a relationship between plasma fibrinolytic parameters and future cardiovascular events, such as stroke or myocardial infarction [6–9]. However, the capacity of endothelial cells to release t-PA from intracellular storage pools, and the rapidity with which this can be mobilised, may not be reflected in the basal circulating plasma concentrations of t-PA antigen or activity [10].

Endothelial cell culture techniques have limitations in the investigation of t-PA release and may not be truly representative of the in vivo function of these cells. The amount of t-PA released in culture is small and necessi-

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tates prolonged incubation periods and sensitive assays. Moreover, the phenotype of endothelial cells in culture, and the ability to release t-PA, changes with increasing passages. In contrast, under in vivo physiological conditions, the endothelium is arranged within a non-planar three-dimensional vascular bed, has a more favourable volume to surface area ratio, and is exposed to pulsatile blood flow and pressure changes. We have recently described an in vivo model to assess acute t-PA release in man [11]. Using intra-brachial infusions of substance P, we have shown a dose-dependent release of t-PA from the human forearm without causing significant release of von Willebrand factor (vWf) or plasminogen activator inhibitor type 1 (PAI-1). This suggests either a selective action of substance P or the lack of a rapidly translocatable pool of PAI-1 and vWf. However, we have previously used only brief (~ 10 min) substance P infusions [11] and protracted stimulation may release these factors [12–14].

Substance P causes endothelium dependent vasodilatation [15] which is mediated by the endothelial cell neurokinin type 1 receptor [16] and is, in part, related to the release of nitric oxide [17–19]. However, because t-PA release is not seen with infusions of the nitric oxide donor and vasodilator, sodium nitroprusside [11,20], an increase in nitric oxide and blood flow together do not release t-PA from the endothelium. Nevertheless, it remains a possibility that the L-arginine/nitric oxide pathway contributes to substance P-induced t-PA release.

Therefore, the aims of the current study were two-fold: first, to ascertain whether prolonged substance P infusion can cause vWf or PAI-1 release; and second, to determine whether nitric oxide synthase inhibition using L-N<sup>G</sup>-monomethylarginine (L-NMMA) affects basal or substance P-induced t-PA release.

## 2. Methods

### 2.1. Subjects

Eight healthy men aged between 20 and 33 years participated in three studies which were undertaken with the approval of the local research ethics committee and in accordance with the Declaration of Helsinki. The written informed consent of each subject was obtained before entry into the study. None of the subjects received vasoactive or non-steroidal anti-inflammatory drugs in the week before each phase of the study, and all abstained from alcohol for 24 h, and from food, tobacco and caffeine-containing drinks for at least 5 h, before each study. All studies were performed in a quiet, temperature-controlled room maintained at 23.5–24.5°C.

### 2.2. Intra-arterial administration and drugs

The brachial artery of the non-dominant arm was cannulated with a 27-standard wire gauge steel needle (Cooper's

Needle Works, Birmingham, UK) under 1% lignocaine (Xylocaine; Astra Pharmaceuticals, Kings Langley, UK) local anaesthesia. The cannula was attached to a 16-gauge epidural catheter (Portex, Hythe, UK) and patency maintained by infusion of saline (0.9%; Baxter Healthcare, Thetford, UK) via an IVAC P1000 syringe pump (IVAC, Basingstoke, UK). The total rate of intra-arterial infusions was maintained constant throughout all studies at 1 ml/min. Pharmaceutical-grade substance P (Clinalfa, Läufelfingen, Switzerland) and L-N<sup>G</sup>-monomethylarginine (L-NMMA; Clinalfa) were administered following dissolution in saline.

### 2.3. Forearm blood flow and blood pressure

Blood flow was measured in both forearms by venous occlusion plethysmography using mercury-in-silastic strain gauges applied to the widest part of the forearm [21]. During measurement periods, the hands were excluded from the circulation by rapid inflation of the wrist cuffs to a pressure of 220 mmHg using E20 Rapid Cuff Inflators (D.E. Hokanson, Washington, USA). Upper arm cuffs were inflated intermittently to 40 mmHg for 10 s in every 15 s to achieve venous occlusion and obtain plethysmographic recordings. Analogue voltage output from an EC-4 Strain Gauge Plethysmograph (D.E. Hokanson) was processed by a MacLab analogue-to-digital converter and Chart v3.3.8 software (AD Instruments, Castle Hill, Australia) and recorded onto a MacIntosh Classic II computer (Apple Computers, Cupertino, USA). Calibration was achieved using the internal standard of the plethysmograph.

Blood pressure was monitored in the non-infused arm at intervals throughout each study using a semi-automated non-invasive oscillometric sphygmomanometer [22] (Takeda UA 751, Takeda Medical, Tokyo, Japan).

### 2.4. Venous sampling and assays

Venous cannulae (17-gauge) were inserted into large subcutaneous veins of the antecubital fossa in both arms as described previously [23]. Ten ml of blood was withdrawn simultaneously from each arm and collected into acidified buffered citrate (Biopool Stabilyte, Umeå, Sweden; for t-PA assays) and citrate (Monovette, Sarstedt, Nümbrecht, Germany; for PAI-1 assays) tubes, and kept on ice before being centrifuged at 2000 g for 30 min at 4°C. Platelet-free plasma was decanted and stored at –80°C before assay.

Plasma PAI-1 and t-PA antigen concentrations were determined using an enzyme-linked immunosorbent assay (ELISA); Coaliza PAI-1 [24] and Coaliza t-PA [25] (Chromogenix AB, Mölndal, Sweden) respectively. Plasma PAI-1 and t-PA activities were determined by a photometric method, Coatest PAI-1 [26] and Coaset t-PA [27] (Chromogenix). Intra-assay coefficients of variation were 7.0



and 5.5% for t-PA and PAI-1 antigen, and 4.0 and 2.4% for activity, respectively. Inter-assay coefficients of variability were 4.0, 7.3, 4.0 and 7.6%, respectively. The sensitivities of the assays were 2.5 ng/ml, 0.5 ng/ml, 5 AU/ml and 0.10 IU/ml, respectively. vWf antigen was determined [28] using an ELISA (Dako, Glostrup, Denmark) with a sensitivity of 0.05 IU/ml. The intra-assay and inter-assay coefficients of variability were 5.2 and 7.3%, respectively. Factor VIII:C procoagulant activity was determined using a standard one-stage assay on an ACL-3000 + coagulometer (Instrumentation Laboratory, Warrington, UK). Haematocrit was determined by capillary tube centrifugation of blood anticoagulated by ethylene diamine tetraacetic acid and was obtained from the infused forearm at baseline and at 120 min.

### 2.5. Study design

On 3 separate occasions, at 09.00 h, subjects attended fasted and rested recumbent throughout each study. Strain gauges and cuffs were applied and the brachial artery of the non-dominant arm cannulated. Throughout all protocols, measurements of forearm blood flow were made every 10 min. Saline was infused for the first 30 min to allow time for equilibration and the final blood flow measurement during saline infusion was taken as the basal forearm blood flow. Thereafter, subjects underwent the following protocols, in random order, each separated by at least 1 week: protocol 1, each subject received intra-arterial substance P at 2, 4 and 8 pmol/min, for 10 min at each dose, followed by a continuous infusion of 8 pmol/min for a further 90 min; protocol 2, L-NMMA was co-infused at 4  $\mu$ mol/min for 10 min before and throughout the same substance P infusion as protocol 1; and protocol 3, subjects received intra-arterial L-NMMA at 1, 2 and 4  $\mu$ mol/min for 10 min at each dose followed by a continuous infusion of 4  $\mu$ mol/min for a further 90 min. Venous samples were withdrawn from each arm at baseline and at 10, 20, 30, 50, 80 and 120 min after the start of substance P (for protocols 1 and 2) or L-NMMA infusion (protocol 3).

### 2.6. Data analysis and statistics

Plethysmographic data were extracted from the Chart data files and forearm blood flows were calculated for individual venous occlusion cuff inflations by use of a template spreadsheet (Excel v4.0; Microsoft, Cambridge, USA). Recordings from the first 60 s after wrist-cuff inflation were not used because of the reflex vasoconstriction this causes [21]. Usually, the last five flow recordings in each 3-min measurement period were calculated and averaged for each arm. Estimated net release of t-PA activity and antigen was defined previously [11] as the product of the infused forearm plasma flow (based on the mean haematocrit, Hct, and the infused forearm blood flow, FBF) and the concentration difference between the infused ( $[t-PA]_{inf}$ ) and non-infused arms ( $[t-PA]_{non-inf}$ ).

Estimated net t-PA release

$$= FBF \times \{1 - Hct\} \times \{[t-PA]_{inf} - [t-PA]_{non-inf}\}$$

Data were examined, where appropriate, by two-way analysis of variance (ANOVA) with repeated measures and two-tailed paired Student's *t*-test using Excel v4.0 (Microsoft). Tachyphylaxis was assessed by comparing the 30-min (peak) and 120-min (final) values with a two-tailed paired Student's *t*-test. Area under the curve (AUC) was calculated for the estimated net release of t-PA across the study period. All results are expressed as mean  $\pm$  s.e.m. Statistical significance was taken at the 5% level.

### 3. Results

All subjects were normotensive and there were no significant changes in blood pressure, heart rate or blood flow in the contralateral arm throughout any of the studies (Table 1). Haematocrit decreased slightly in each study (Table 1). Between the 3 protocols, there were no significant differences in the baseline values of blood pressure,

Table 1  
Haemodynamics and haematocrit at baseline and completion of the 3 study protocols

	Substance P alone		L-NMMA alone		Substance P + L-NMMA	
	Basal	Final	Basal	Final	Basal	Final
Blood pressure (mmHg)						
Systolic	137 $\pm$ 3	136 $\pm$ 3	135 $\pm$ 5	136 $\pm$ 5	133 $\pm$ 6	132 $\pm$ 6
Diastolic	71 $\pm$ 2	71 $\pm$ 3	68 $\pm$ 3	71 $\pm$ 3	69 $\pm$ 4	70 $\pm$ 5
Heart rate (/min)	65 $\pm$ 4	61 $\pm$ 3	59 $\pm$ 2	58 $\pm$ 3	60 $\pm$ 3	61 $\pm$ 3
Absolute forearm blood flow (ml/100 ml/min)						
Non-infused arm	3.1 $\pm$ 0.3	3.7 $\pm$ 0.5	3.4 $\pm$ 0.7	3.6 $\pm$ 0.6	3.6 $\pm$ 0.5	4.9 $\pm$ 0.7
Infused arm	3.6 $\pm$ 0.4	12.1 $\pm$ 1.3 <sup>a</sup>	3.9 $\pm$ 0.7	2.1 $\pm$ 0.2	4.2 $\pm$ 0.7	9.3 $\pm$ 1.4 <sup>a</sup>
Haematocrit	0.439 $\pm$ 0.006	0.430 $\pm$ 0.007 <sup>b</sup>	0.430 $\pm$ 0.010	0.421 $\pm$ 0.013 <sup>c</sup>	0.426 $\pm$ 0.008	0.414 $\pm$ 0.010 <sup>b</sup>

<sup>a</sup>*P* < 0.001.

<sup>b</sup>*P* < 0.005.

<sup>c</sup>*P* = 0.02.

heart rate, forearm blood flow, haematocrit or plasma concentrations of t-PA and PAI-1 antigen and activity.

### 3.1. Isolated infusions of substance P and L-NMMA

Substance P increased blood flow in the infused arm ( $P < 0.001$ ) in a dose-dependent manner (Fig. 1 and Table 2) reaching a maximum increase of  $15.9 \pm 1.9$  ml/100 ml/min after 10 min at 8 pmol/min. Following prolonged infusion, substance P-induced vasodilatation demonstrated tachyphylaxis and decreased to  $12.1 \pm 1.3$  ml/100 ml/min after 100 min of substance P at 8 pmol/min ( $P < 0.003$  vs. 10 min). In comparison to the non-infused arm, substance P caused a dose-dependent increase in venous plasma t-PA activity ( $P < 0.001$ ) and antigen ( $P < 0.04$ ) concentrations of the infused arm which did not undergo

significant tachyphylaxis (Fig. 2). Concentrations of plasma PAI-1 activity were also reduced in the infused arm ( $P = 0.04$ ; Fig. 2). In contrast, there were no significant changes in plasma PAI-1 antigen, vWf or factor VIII:C concentrations in either arm (Fig. 2 and Table 2).

L-NMMA decreased blood flow in the infused arm ( $P < 0.001$ ) in a dose-dependent manner (Fig. 1 and Table 2) reaching  $2.1 \pm 0.2$  ml/100 ml/min after 100 min at 4  $\mu$ mol/min. There were no significant changes in the concentrations of plasma t-PA and PAI-1 antigen or activity in either arm during infusion of L-NMMA (Fig. 2).

### 3.2. Co-infusion of L-NMMA and substance P

In the presence of L-NMMA, substance P increased blood flow in the infused arm ( $P < 0.001$ ) in a dose-de-

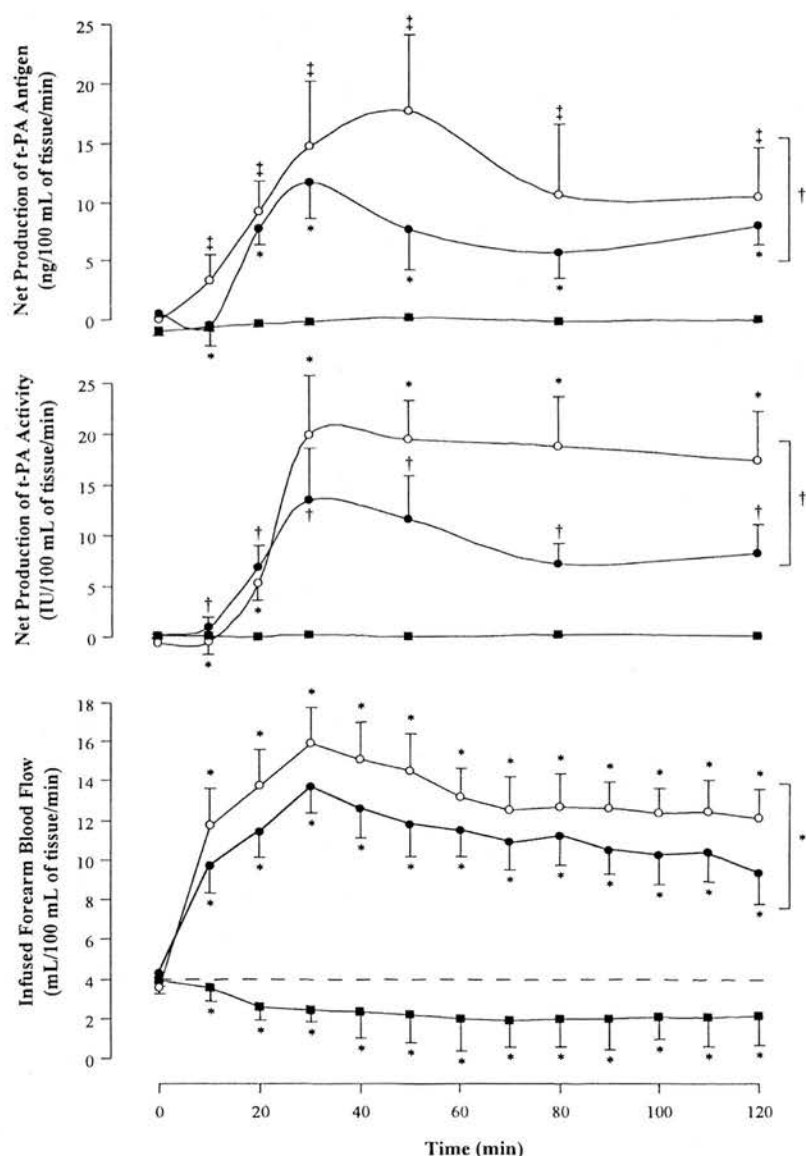


Fig. 1. Infused forearm blood flow and estimated net release of t-PA antigen and activity during protocol 1 (substance P alone, O), protocol 2 (substance P and L-NMMA, ●) and protocol 3 (L-NMMA alone; ■). \*  $P < 0.001$ ; †  $P \leq 0.05$ ; ‡  $P = 0.09$  (ANOVA).

Table 2

Blood flow and plasma von Willebrand's factor and factor VIII:C activity concentrations in both arms during isolated substance infusion: protocol 1

	Baseline	Time (min)					
		10	20	30	50	80	120
Substance P dose (pmol/min)	0	2	4	8	8	8	8
Absolute forearm blood flow (ml/100 ml/min)							
Non-infused arm	3.1 ± 0.3	3.3 ± 0.3	3.4 ± 0.4	3.3 ± 0.3	3.5 ± 0.3	3.8 ± 0.4	3.7 ± 0.5
Infused arm	3.6 ± 0.4	11.8 ± 1.8	13.8 ± 1.9	15.9 ± 1.9	14.5 ± 1.7	12.6 ± 1.2	12.1 ± 1.3 <sup>a</sup>
von Willebrand's factor (IU/ml)							
Non-infused arm	0.84 ± 0.13	0.64 ± 0.10	0.85 ± 0.13	0.60 ± 0.09	0.95 ± 0.18	0.80 ± 0.10	1.20 ± 0.16
Infused arm	0.73 ± 0.09	0.72 ± 0.15	0.73 ± 0.11	0.86 ± 0.20	0.98 ± 0.16	0.99 ± 0.17	1.03 ± 0.17
Factor VIII:C (IU/ml)							
Non-infused arm	0.50 ± 0.06	0.48 ± 0.04	0.51 ± 0.05	0.51 ± 0.05	0.58 ± 0.09	0.65 ± 0.10	0.72 ± 0.09
Infused arm	0.50 ± 0.05	0.50 ± 0.05	0.51 ± 0.07	0.52 ± 0.07	0.64 ± 0.07	0.64 ± 0.08	0.69 ± 0.10

<sup>a</sup> $P < 0.001$ .

pendent manner (Fig. 1) reaching a maximum increase of  $13.7 \pm 1.7$  ml/100 ml/min after 10 min at 8 pmol/min. This response underwent tachyphylaxis and decreased to  $9.3 \pm 1.4$  ml/100 ml/min after 100 min of substance P at 8 pmol/min ( $P < 0.002$  vs. 10 min). In comparison, with the non-infused arm, substance P co-infused with L-NMMA caused a dose-dependent increase in plasma t-PA activity ( $P < 0.001$ ) and antigen ( $P < 0.003$ ) concentrations of the infused arm which did not undergo significant tachyphylaxis (Fig. 2). L-NMMA caused a significant attenuation of

substance P-induced increases in blood flow ( $P < 0.001$ ) and plasma t-PA activity concentrations ( $P < 0.003$ ) in the infused forearm, but not plasma t-PA antigen.

### 3.3. Estimated net t-PA production

L-NMMA infused alone had no significant effects on t-PA release: 95% confidence intervals for t-PA antigen and activity release are 0.31 to  $-0.68$  ng/100 ml/min and 0.27 to  $-0.06$  IU/100 ml/min, respectively. Sub-

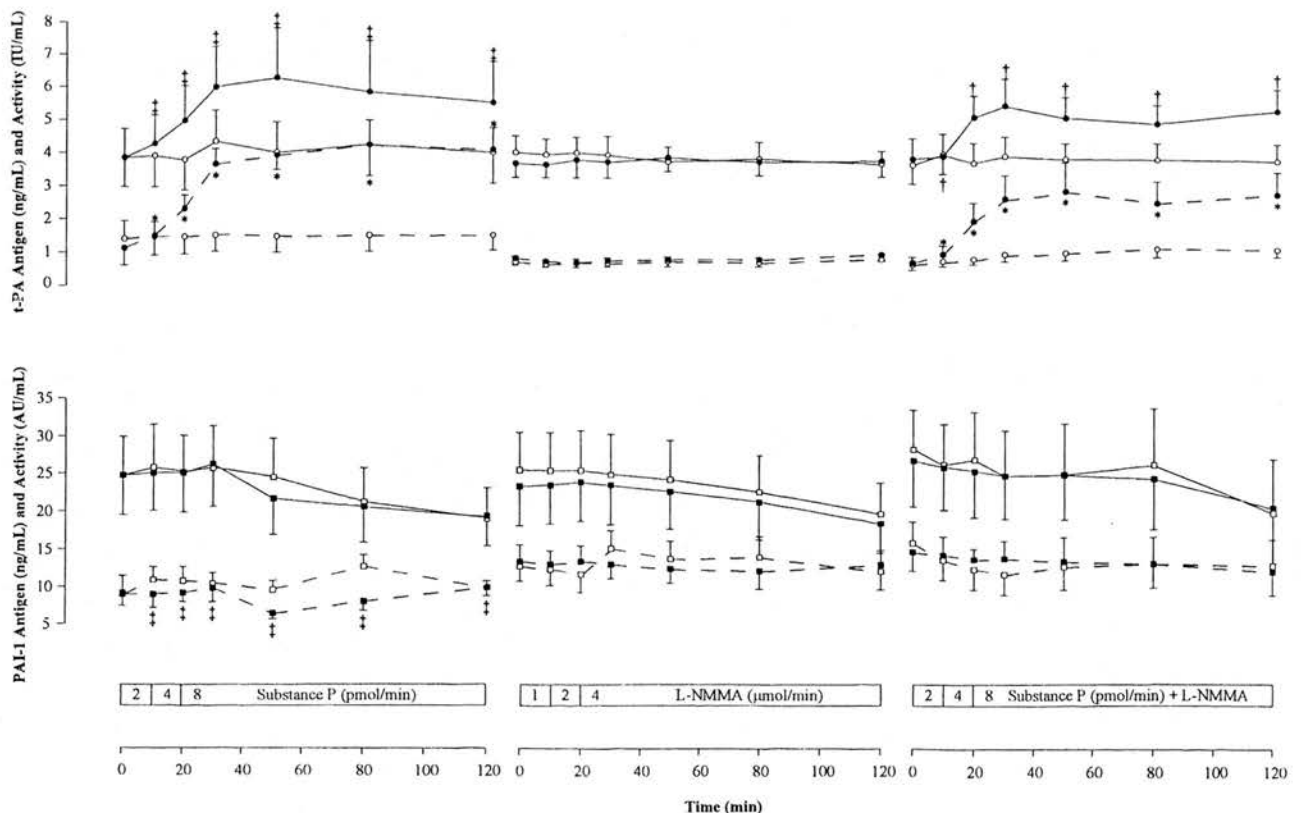


Fig. 2. Plasma concentrations of t-PA and PAI-1 antigen (solid lines) and activity (dashed lines) in the infused (● and ■, respectively) and non-infused (○ and □, respectively) arms in the 3 protocols. \*  $P < 0.001$ ; †  $P < 0.003$ ; ‡  $P \leq 0.04$  (ANOVA).



stance P caused dose-dependent increases in the estimated net release of t-PA antigen and activity in the presence or absence of L-NMMA ( $P < 0.001$ ) which did not undergo significant tachyphylaxis. However, the magnitude of the increase in release of both t-PA antigen ( $P = 0.05$ ) and activity ( $P < 0.01$ ) was significantly reduced in the presence of L-NMMA (Fig. 1). L-NMMA reduced the AUC for the substance P-induced release of t-PA antigen and activity by 40 and 46% respectively.

#### 4. Discussion

We have shown that intra-brachial substance P infusion increases forearm blood flow and plasma t-PA concentrations for up to 2 h without a demonstrable effect on plasma PAI-1 or vWf concentrations. Although the nitric oxide synthase inhibitor, L-NMMA, significantly reduced forearm blood flow without affecting basal t-PA release, it inhibited the increases in blood flow, plasma t-PA concentrations and t-PA release produced by substance P administration in the forearm. These data suggest that the L-arginine/nitric oxide pathway contributes to substance P-induced t-PA release *in vivo* in man. In contrast, we [11] and others [20] have shown previously that t-PA release is not seen with the large local increases in nitric oxide delivery and blood flow associated with infusions of the nitric oxide donor, sodium nitroprusside. Taken together, these findings indicate that increases in nitric oxide and blood flow are not sufficient *per se* to release t-PA but, through the L-arginine/nitric oxide pathway, are able to enhance substance P-induced t-PA release.

The permissive role of intracellular mediators in the mechanism of t-PA release has been described previously. In the rat perfused hindlimb model, increasing intracellular calcium alone is insufficient to cause t-PA release whilst it is essential for bradykinin induced t-PA release [29]. However, the regulation of t-PA release is complex and may involve several signal transduction pathways [30]. This is reflected by the diversity of mediators, such as thrombin, bradykinin and desmopressin, which can release t-PA and increase t-PA activity [12,13,31,32]. One can, therefore, only speculate as to whether our findings extend to the acute t-PA release seen with *in situ* thrombosis. However, both nitric oxide mediated endothelial dysfunction [33–36] and abnormalities of endogenous fibrinolysis [6–9] have been described in many atherosclerotic diseases and the associated risk factors. Thus, the coupling of acute t-PA release to the L-arginine/nitric oxide pathway provides an important potential mechanism whereby endothelial dysfunction might increase the risk of atherothrombosis through a reduction in the acute fibrinolytic capacity. Our initial findings would suggest that this model could be applied to the assessment of the acute fibrinolytic capacity of patients with endothelial dysfunction such as those with hypercholesterolaemia and a smoking habit [35,36], and to

the examination of the subsequent effect of L-arginine supplementation.

Substance P-induced vasodilatation undergoes tachyphylaxis [37] which may relate to internalisation of the neurokinin type 1 receptor from the endothelial cell surface membrane [38]. It has been suggested from *ex vivo* animal studies [15,39] that the residual vasodilatation following the development of tachyphylaxis is almost completely nitric oxide-dependent. In the present study, the degree of inhibition of substance P-induced vasodilatation by L-NMMA was less than we [18] and others [17] have previously described and may reflect the higher potency and doses used in this study. Whilst we have readily demonstrated tachyphylaxis of substance P-induced vasodilatation, the co-infusion of L-NMMA did not affect the development of tachyphylaxis and did not abolish the residual substance P-induced vasodilatation following its development. Thus, in contrast to animal studies, residual vasodilatation after the development of tachyphylaxis does not appear to be predominantly nitric oxide mediated in the human forearm. In addition, we were unable to detect significant tachyphylaxis of substance P-induced increases in plasma t-PA antigen and activity concentrations, suggesting that not all the actions of substance P undergo tachyphylaxis.

The substance P-induced reductions in plasma PAI-1 activity of the infused arm without significant alterations in PAI-1 antigen concentrations are consistent with acute t-PA release in the absence of PAI-1 release [40]. PAI-1 binds to the newly released t-PA to form an inactive PAI-1/t-PA complex, thereby reducing the plasma PAI-1 activity. The trend for PAI-1 antigen concentrations to fall in both arms as the study progressed is consistent with systemic (hepatic) clearance of the PAI-1/t-PA complex [40–42]. However, this trend was also seen with isolated L-NMMA infusion in which there was no significant release of t-PA consistent with a circadian fall of PAI-1 antigen during the morning [43].

Despite reducing forearm blood flow by half, L-NMMA did not significantly affect the constitutive release or plasma concentrations of t-PA and PAI-1 antigen and activity. The 95% confidence intervals indicates that if L-NMMA has an effect on basal t-PA or PAI-1 release then it is rather small. This suggests that the L-arginine/nitric oxide pathway does not play a major role in the basal release of t-PA or PAI-1 in the peripheral vasculature of man.

##### 4.1. Study limitations

Since the derivation of t-PA release is a function of plasma flow, it could be argued that the inhibition by L-NMMA of substance P-induced t-PA release reflects the simultaneous reduction in blood flow. However, the reduction in absolute blood flow was only modest (15–20%) in comparison to the reduction in t-PA release (40–46%) and the plasma t-PA activity concentrations in the infused

forearm were also significantly reduced by co-infusion of L-NMMA. The findings of the present study would be strengthened by utilising a control vasoconstrictor and demonstrating a neutral effect on substance P-induced t-PA release. However, standard receptor coupled vasoconstrictors used in forearm studies, such as noradrenaline, vasopressin and angiotensin II, are known to stimulate t-PA and PAI-1 release [12,14,32,44] and would not help in interpreting the influence of L-NMMA on substance P-induced t-PA release.

We have previously been unable to detect an acute local release of either vWf or PAI-1 during 10-min infusions of substance P given at 8-fold higher concentrations [11]. In the present study, substance P did not cause significant vWf or PAI-1 release, despite infusion times of up to 120 min, suggesting that the dissociation of substance P-induced t-PA release from vWf is not a temporal effect. However, this dissociated release does not appear to be unique to substance P since this has also been recently described with local forearm infusions of desmopressin [45]. These findings are, however, limited to the peripheral forearm vascular bed and the endothelium in other tissue beds may respond differently to substance P stimulation. The extension of this model to vascular beds associated with atherosclerosis such as the coronary circulation, will be of crucial relevance in determining the influence of atheroma and endothelial dysfunction on the acute local release of t-PA during thrombotic occlusion and plaque rupture.

In summary, in the forearm vascular bed in vivo, we have shown for the first time that the L-arginine/nitric oxide pathway contributes to substance P-induced t-PA release in man. This coupling of acute t-PA release to the L-arginine/nitric oxide pathway provides an important potential mechanism whereby endothelial dysfunction increases the risk of atherothrombosis through a reduction in the acute fibrinolytic capacity.

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## **PUBLICATION 3**

# Endothelin-1 does not contribute to the release of tissue plasminogen activator in vivo in man

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**Summary Objectives:** Endothelin-1 is a potent endothelium-derived vasoconstrictor peptide with autocrine and paracrine actions. Tissue plasminogen activator (t-PA) and its inhibitor, plasminogen activator inhibitor type 1 (PAI-1), are also released from the vascular endothelium and play a pivotal role in endogenous fibrinolysis. We, therefore, examined the effects of exogenous and endogenous endothelin-1 on t-PA and PAI-1 release in vivo in man.

**Design:** Open investigative study.

**Setting:** Clinical Research Centre, University of Edinburgh.

**Subjects:** Fourteen healthy male volunteers.

**Interventions:** Unilateral brachial artery infusions of endothelin-1 at 2.5 and 10 pmol/min, and the selective endothelin type B (ET<sub>B</sub>) receptor antagonist, BQ-788, at 1 nmol/min.

**Main outcome measures:** Blood flow and plasma fibrinolytic factors were measured in both forearms using venous occlusion plethysmography and venous blood samples withdrawn from the antecubital fossae.

**Results:** Endothelin-1 caused a slow onset dose-dependent forearm vasoconstriction ( $P < 0.001$ ) with a maximal reduction in blood flow of  $40 \pm 4\%$  and  $63 \pm 3\%$  at 2.5 and 10 pmol/min respectively. BQ-788 also caused a slow onset reduction in forearm blood flow ( $P < 0.001$ ) reaching a maximum of  $21 \pm 3\%$ . However, BQ-788 and endothelin-1 did not affect plasma concentrations of t-PA or PAI-1 in the venous effluent of the infused forearm.

**Conclusions:** Despite sustaining significant vasoconstriction, neither endogenous nor exogenous endothelin-1 influences the release of t-PA or PAI-1 in the forearm vascular bed of man. This suggests that endothelin-1 does not provide a major contribution to the regulation of endogenous fibrinolysis in man. © Harcourt Publishers Ltd 1999

## INTRODUCTION

Endothelial cells in the precapillary arterioles and post-capillary venules<sup>1</sup> synthesize and release t-PA and PAI-1 both basally and in response to stimulation by various coagulation factors and stimulants. The time course of t-PA release is important since clot dissolution is much more effective if t-PA is incorporated during clot formation rather than following completion.<sup>2,3</sup> The acute release of t-PA results from the rapid translocation of a dynamic intracellular storage pool<sup>4</sup> and plays a pivotal role in endogenous fibrinolysis.

Endothelin-1 is a potent endothelium-derived vasoconstrictor peptide with autocrine and paracrine actions. It is continuously released by the endothelium and contributes to the maintenance of basal vascular tone<sup>5</sup> and blood pressure.<sup>6</sup> There are two main endothelin receptor subtypes, ET<sub>A</sub> and ET<sub>B</sub>, but only the ET<sub>B</sub> receptors are present on the endothelium. Endothelin-1 causes vasoconstriction mainly through stimulation of the smooth muscle cell ET<sub>A</sub> receptor, although smooth muscle ET<sub>B</sub> receptors may also contribute in some vessel types. This vasoconstrictor response is modulated by autocrine endothelial cell ET<sub>B</sub> receptor-mediated generation of the endothelium-derived vasodilators, nitric oxide and prostacyclin.

The importance of endogenous t-PA release is exemplified by the high rate of spontaneous reperfusion in the infarct-related artery after acute myocardial infarction, occurring in around 30% of patients within the first 12 h.<sup>7–9</sup>

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Following an acute myocardial infarction, plasma endothelin-1 concentrations are elevated and provide an important prognostic marker of survival at 1 year.<sup>10</sup> Furthermore, on the basis of *in vitro* studies, it has been suggested that endothelin-1 may contribute to the regulation of endogenous fibrinolysis and t-PA release.<sup>11–13</sup> However, the evidence is contradictory, with endothelin-1 being found to either inhibit<sup>13</sup> or stimulate<sup>11,12</sup> endothelial cell t-PA release. The role of endothelin-1 in the regulation of endogenous fibrinolysis in man is currently unknown.

We,<sup>14,15</sup> and others,<sup>16,17</sup> have shown, using bilateral forearm venous occlusion plethysmography and unilateral brachial artery infusions, that the forearm release of t-PA and PAI-1 can be determined *in vivo* in man. Therefore, the aim of the current study was, using synthetic endothelin-1 peptide and the selective ET<sub>B</sub> receptor antagonist, BQ-788, to determine whether endothelin-1, of exogenous or endogenous origin, acts via the endothelial ET<sub>B</sub> receptor to regulate the release of t-PA or PAI-1 *in vivo* in man.

## MATERIALS AND METHODS

### Subjects

Fourteen healthy men aged between 20 and 33 years participated in three studies which were undertaken with the approval of the local research ethics committee and in accordance with the Declaration of Helsinki. The written informed consent of each subject was obtained before entry into the study. None of the subjects received vasoactive or non-steroidal anti-inflammatory drugs in the week before each phase of the study, and all abstained from alcohol for 24 h, and from food, tobacco and caffeine-containing drinks for at least 9 h, before each study. All studies were performed in a quiet, temperature-controlled room maintained at 23.5–24.5°C.

### Intra-arterial administration and drugs

The brachial artery of the non-dominant arm was cannulated with a 27-standard wire gauge steel needle (Cooper's Needle Works Ltd, Birmingham, UK) under 1% lignocaine (Xylocaine; Astra Pharmaceuticals Ltd, Kings Langley, UK) local anaesthesia. The cannula was attached to a 16-gauge epidural catheter (Portex Ltd, Hythe, UK) and patency maintained by infusion of saline (0.9%: Baxter Healthcare Ltd, Thetford, UK) via an IVAC P1000 syringe pump (IVAC Ltd, Basingstoke, UK). The total rate of intra-arterial infusions was kept constant throughout all studies at 1 mL/min. Endothelin-1 (Clinalfa AG, Läufelfingen, Switzerland) and BQ-788 (American Peptide Company, Sunnyvale, USA) were administered following dissolution in saline.

### Forearm blood flow and blood pressure

Blood flow was measured in both forearms by venous occlusion plethysmography using mercury-in-silastic strain gauges applied to the widest part of the forearm.<sup>18,19</sup> During measurement periods the hands were excluded from the circulation by rapid inflation of the wrist cuffs to a pressure of 220 mmHg using E20 Rapid Cuff Inflators (D.E. Hokanson Inc, Washington, USA). Upper arm cuffs were inflated intermittently to 40 mmHg for 10 s in every 15 s to achieve venous occlusion and obtain plethysmographic recordings. Analogue voltage output from an EC-4 Strain Gauge Plethysmograph (D.E. Hokanson) was processed by a MacLab<sup>®</sup> analogue-to-digital converter and Chart v3.3.8 software (AD Instruments Ltd, Castle Hill, Australia) and recorded onto a Macintosh Classic II computer (Apple Computers Inc, Cupertino, USA). Calibration was achieved using the internal standard of the plethysmograph.

Blood pressure was monitored in the non-infused arm at intervals throughout each study using a semi-automated non-invasive oscillometric sphygmomanometer (Takeda UA 751, Takeda Medical Inc, Tokyo, Japan).<sup>20</sup>

### Venous sampling and assays

Venous cannulae (17G) were inserted into large subcutaneous veins of the antecubital fossa in both arms. Ten mL of blood was withdrawn simultaneously from each arm and collected into acidified buffered citrate (Biopool<sup>®</sup> Stabilyte™, Umeå, Sweden; for t-PA assays) and citrate (Monovette<sup>®</sup>, Sarstedt, Nümbrecht, Germany; for PAI-1 assays) tubes, and kept on ice before being centrifuged at 2000 g for 30 min at 4°C. Platelet-free plasma was decanted and stored at –80°C before assay.<sup>21</sup>

Plasma PAI-1 and t-PA antigen concentrations were determined using an enzyme-linked immunosorbent assay (ELISA); Coaliza<sup>®</sup> PAI-1 [22] and Coaliza<sup>®</sup> t-PA<sup>23</sup> (Chromogenix AB, Mölndal, Sweden) respectively. Plasma t-PA activities were determined by a photometric method, Coaset<sup>®</sup> t-PA<sup>24</sup> (Chromogenix AB). Intra-assay coefficients of variation were 7 and 5.5% for t-PA and PAI-1 antigen, and 4% for t-PA activity respectively. Inter-assay coefficients of variability were 4, 7.3 and 4% respectively. The sensitivities of the assays were 2.5 ng/mL, 0.5 ng/mL and 0.10 IU/mL respectively. Haematocrit was determined by capillary tube centrifugation of blood anticoagulated by ethylene diamine tetraacetic acid and was obtained from the infused forearm at baseline and at 120 min.

### Study design

On each study day, subjects attended fasted and rested recumbent throughout the study. Strain gauges and cuffs

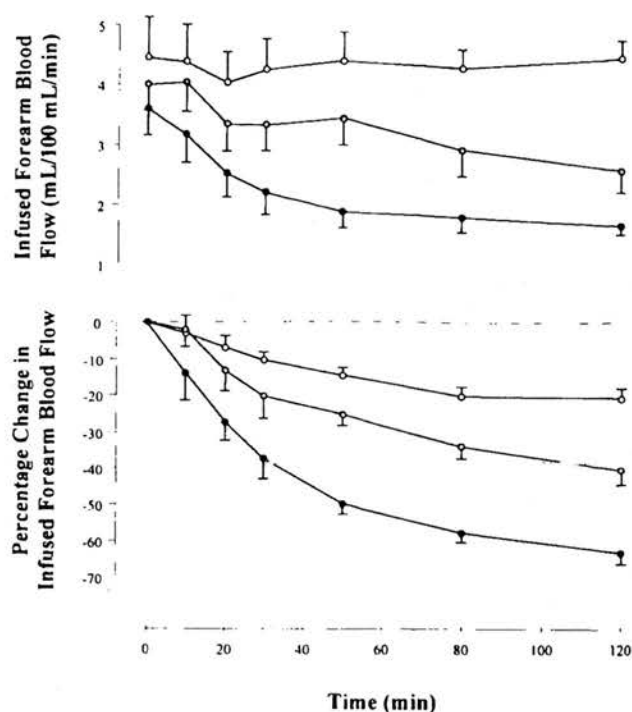


Fig. 1 Absolute (mL/100 mL of tissue/min; upper panel) and percentage (% relative to the non-infused forearm; lower panel) change of blood flow in the infused forearm during intra-arterial infusion of BQ-788 (1 nmol/min; ○) and endothelin-1 (2.5 pmol/min; ○ and 10 pmol/min; ●).

were applied and the brachial artery of the non-dominant arm cannulated. Throughout each of the studies, measurements of forearm blood flow were made every 10 min. Before drug administration, saline was infused for 30 min to allow time for equilibration and the final blood flow measurement during saline infusion was taken as the basal forearm blood flow.

Eight subjects received an intra-brachial infusion of endothelin-1 at 2.5 and 10 pmol/min for 120 min, given in random order, on two separate occasions, at least 1 week apart. Eight subjects (two had also attended for endothelin-1 infusions) received an intra-brachial infusion of BQ-788 at 1 nmol/min for 120 min. Venous samples were withdrawn from each arm at baseline and at 10, 20, 30, 50, 80 and 120 min after the start of endothelin-1 or BQ-788 infusion.

#### Data analysis and statistics

Plethysmographic data were extracted from the Chart data files and forearm blood flows were calculated for individual venous occlusion cuff inflations by use of a template spreadsheet (Excel v5; Microsoft Corporation, Cambridge, USA). Recordings from the first 60 s after wrist cuff inflation were not used because of the reflex vasoconstriction this causes.<sup>18,19</sup> Usually, the last five flow recordings in each 3 min measurement period were calculated and averaged for each arm. To reduce the variability of blood flow data, the ratio of flows in the two arms was calculated for each time point: in effect using the non-infused arm as a contemporaneous control for the infused arm.<sup>18,19</sup> Percentage changes in the infused forearm blood flow were calculated<sup>18,19</sup> as follows:

$$\% \text{ Change in blood flow} = \frac{100 \times (I_t/NI_t - I_b/NI_b)}{I_b/NI_b}$$

Where  $I_b$  and  $NI_b$  are the infused and non-infused forearm blood flows at baseline (time 0) respectively, and  $I_t$  and  $NI_t$  are the infused and non-infused forearm blood flows at a given time point respectively.

Estimated net release of t-PA activity and antigen was defined previously<sup>14,15</sup> as the product of the infused forearm plasma flow (based on the mean haematocrit, Hct,

Table 1 Systemic haemodynamics, forearm blood flow and haematocrit at baseline and after intra-arterial infusion for 120 min.

	BQ-788 1 nmol/min		Endothelin-1 2.5 pmol/min		Endothelin-1 10 pmol/min	
	Basal	Final	Basal	Final	Basal	Final
Blood pressure (mmHg)						
systolic	130 ± 5	134 ± 6	136 ± 3	140 ± 4	133 ± 4	133 ± 4
diastolic	75 ± 4	77 ± 4	72 ± 3	71 ± 3	70 ± 3	73 ± 4
Heart rate (/min)	59 ± 3	60 ± 3	62 ± 4	58 ± 4	61 ± 5	62 ± 4
Absolute forearm blood flow (mL/100 mL/min)						
non-infused arm	3.1 ± 0.4	3.8 ± 0.2	3.4 ± 0.3	3.7 ± 0.5	3.1 ± 0.3	4.1 ± 0.3
infused arm	4.5 ± 0.7	4.2 ± 0.4*	4.0 ± 0.4	2.5 ± 0.3*	3.6 ± 0.4	1.6 ± 0.1*
Ratio of infused/non-infused	1.35 ± 0.15	1.14 ± 0.06†	1.13 ± 0.03	0.42 ± 0.03†	1.05 ± 0.07	0.40 ± 0.03†
Haematocrit	0.41 ± 0.01	0.41 ± 0.01	0.42 ± 0.02	0.40 ± 0.02‡	0.41 ± 0.01	0.40 ± 0.01‡

\* $P \leq 0.001$  (two-way ANOVA; infused vs non-infused)

† $P < 0.001$  (one-way ANOVA)

‡ $P < 0.05$  (paired t-test; basal vs final)

and the infused forearm blood flow, FBF) and the concentration difference between the infused ([t-PA]<sub>inf</sub>) and non-infused arms ([t-PA]<sub>Non-inf</sub>).

Estimated net t-PA release =  

$$FBF \times \{1-HCt\} \times \{[t-PA]_{inf} - [t-PA]_{Non-inf}\}$$

Data were examined by two way analysis of variance (ANOVA) with repeated measures and two-tailed paired Student's t-test using Excel v5.0 (Microsoft) where appropriate. All results are expressed as mean ± SEM. Statistical significance was taken at the 5% level. Based on previous data,<sup>14,15</sup> the study had 90% power to detect a 20% change in plasma t-PA concentrations between treatment periods at the 5% level.

RESULTS

All subjects were normotensive and there were no significant changes in blood pressure, heart rate or blood flow in the contralateral arm throughout any of the studies (Table 1). Haematocrit decreased slightly in each endothelin study (Table 1). Between the 3 protocols there were no significant differences in the baseline values of blood pressure, heart rate, forearm blood flow, haematocrit or plasma concentrations of t-PA and PAI-1.

Endothelin-1 infusions

Endothelin-1 decreased blood flow in the infused arm (*P*<0.001) in a dose-dependent manner (Fig. 1) reaching a minimum of 2.5 ± 0.3 mL/100 mL/min at 2.5 pmol/min and 1.6 ± 0.1 mL/100 mL/min at 10 pmol/min, after 120 min. This corresponds to a relative reduction in forearm blood flow of 40 ± 4% and 63 ± 3% respectively. The plasma concentrations of t-PA and PAI-1 did not change in the infused arm (Fig. 2) during endothelin-1 infusion at either concentration (*P*=NS; one-way ANOVA). In comparison to the non-infused arm, there was a trend (*P*=0.06; two-way ANOVA) for the infused forearm plasma t-PA antigen concentration to be greater with 10

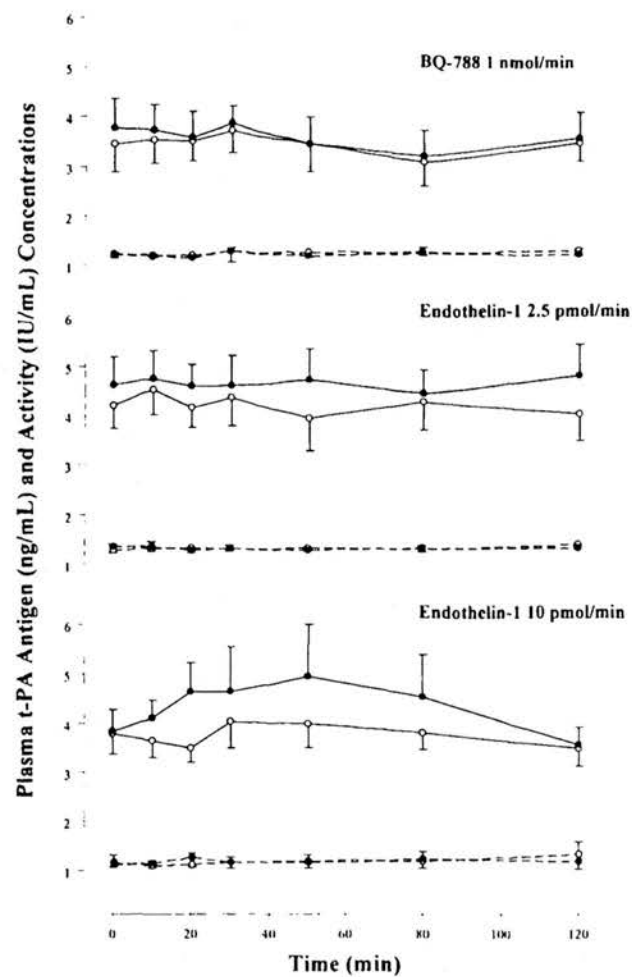


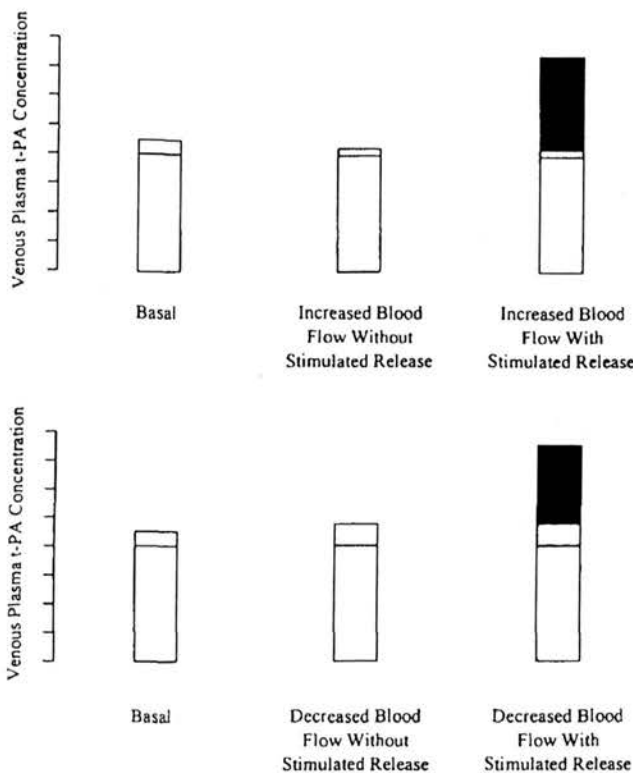
Fig. 2 Plasma concentrations of tissue plasminogen activator (t-PA) antigen (ng/mL; solid lines) and activity (IU/mL; dashed lines) in the infused (solid circles) and non-infused (open circles) forearm during intra-arterial infusion of BQ-788 (1 nmol/min) and endothelin-1 (2.5 and 10 pmol/min).

pmol/min of endothelin-1. However, there were no significant differences in plasma concentrations of PAI-1 antigen (Table 2) or t-PA activity between the forearms.

Table 2 Plasma plasminogen activator inhibitor type 1 (PAI-1) concentrations (ng/mL) during endothelin-1 (ET-1) and BQ-788 infusion. Mean ± SEM.

	Time (min)						
	Baseline	10	20	30	50	80	120
BQ-788 (1 nmol/min)							
infused arm	39 ± 15	37 ± 13	43 ± 15	41 ± 13	37 ± 12	37 ± 12	29 ± 9
non-infused arm	38 ± 13	40 ± 14	43 ± 14	41 ± 13	37 ± 13	33 ± 9	32 ± 9
ET-1 (2.5 pmol/min)							
infused arm	28 ± 7	27 ± 5	26 ± 6	26 ± 5	25 ± 5	21 ± 5	20 ± 5
non-infused arm	27 ± 5	29 ± 6	29 ± 6	27 ± 5	28 ± 7	25 ± 5	23 ± 4
ET-1 (10 pmol/min)							
infused arm	25 ± 5	26 ± 4	27 ± 5	26 ± 5	23 ± 5	21 ± 4	22 ± 5
non-infused arm	27 ± 5	27 ± 5	26 ± 5	25 ± 4	24 ± 5	23 ± 5	22 ± 4





**Fig. 3** Theoretical components of venous plasma t-PA concentration under basal conditions and during increases (upper panel) and decreases (lower panel) in blood flow with and without direct stimulation of t-PA release. Open bars: circulating or arterial t-PA; grey bars: basal 'constitutive' t-PA released from the tissue bed; black bars: stimulated 'facultative' t-PA released from the tissue bed

### BQ-788 infusion

In comparison to the non-infused arm, BQ-788 decreased blood flow in the infused forearm after 120 min (relative reduction of  $21 \pm 3\%$ ) although the absolute blood flow was unchanged (Fig. 1 and Table 1). The plasma concentrations of t-PA and PAI-1 (Fig. 2 and Table 2) did not change in the infused forearm ( $P=NS$ ; one-way ANOVA) or in comparison to the non-infused forearm ( $P=NS$ ; two-way ANOVA).

There was no significant net release of t-PA with infusions of either endothelin-1 or BQ-788 (Table 3).

### DISCUSSION

We have demonstrated that, despite causing significant reductions in blood flow, neither endogenous nor exogenous endothelin-1 influences the release of t-PA or PAI-1 in the forearm vascular bed of man. This suggests that endothelin-1 does not contribute to the regulation of endogenous fibrinolysis in man.

Endothelial cell culture techniques have limitations in the investigation of t-PA release and may not be truly representative of the in vivo function of these cells. The amount of t-PA released in culture is small and necessitates prolonged incubation periods and sensitive assays. Moreover, the phenotype of endothelial cells in culture, and the ability to release t-PA, changes with increasing passages. This may account for the disparity of our findings with previous endothelial cell culture studies.<sup>13</sup>

Studies in intact whole animals have suggested that systemic endothelin-1 infusion is associated with stimulation of t-PA release,<sup>12</sup> although plasma t-PA concentrations are not increased by low sub-pressor doses of endothelin-1 in man.<sup>25</sup> Systemic endothelin-1 administration, particularly at pressor doses, will induce changes in cardiac function and regional blood flow as well as having widespread effects on disparate tissues. Thus, the consequent changes in systemic fibrinolytic parameters will be a combination of many factors, potentially including hepatic production and clearance of t-PA and PAI-1. One approach, to avoid these confounding systemic effects, has been to use the isolated perfused rat hindlimb model. This ex vivo model has been reported to demonstrate that endothelin-1 infusion stimulates modest amounts of t-PA release.<sup>11</sup> However, this increased 'release' may, in part, reflect the concentrating effects of a reduction in blood flow associated with endothelin-1 infusion and the concentrations of endothelin-1 administered. In studies conducted to date,<sup>11-13</sup> endothelin-1 has been administered in nanomolar concentrations.

**Table 3** Estimated net release of tissue plasminogen activator (t-PA) antigen across the forearm during endothelin-1 (ET-1) and BQ-788 infusion. Mean (95% confidence intervals)

	Time (min)						
	Baseline	10	20	30	50	80	120
t-PA release (ng/100 mL/min)							
BQ-788 (1 nmol/min)	1.1 (-0.1 to 2.3)	0 (-1.6 to 1.6)	0.4 (-1.0 to 1.8)	0.3 (-1.5 to 2.1)	-0.2 (-1.8 to 1.4)	0.1 (-1.3 to 1.5)	0.1 (-1.7 to 1.9)
ET-1 (2.5 pmol/min)	0.1 (-0.7 to 0.9)	1.4 (-0.6 to 3.4)	2.5 (0.5 to 4.5)	1 (-0.8 to 2.6)	2 (-1.0 to 5.0)	1.7 (-1.1 to 4.5)	0 (-0.2 to 0.2)
ET-1 (10 pmol/min)	1.3 (-0.3 to 2.9)	0.5 (-0.4 to 1.9)	0.9 (0.1 to 1.7)	0.3 (-0.4 to 1.7)	0.9 (-0.1 to 1.9)	0.1 (-0.7 to 0.9)	0.8 (0.0 to 1.6)

Although local abluminal concentrations may be high, normal human plasma endothelin-1 concentrations are in the femtomolar range. Indeed, in the present study, assuming a total forearm blood flow of 30–50 mL/min, the forearm tissue concentration of endothelin-1 during the 10 pmol/min infusion will be 200–300 fmol/mL. The previous *ex vivo* animal studies,<sup>11</sup> therefore, represent some 4–5 orders of magnitude higher concentrations and the release of t-PA is likely to represent a pharmacological rather than physiological effect.

We have not detected a significant release of t-PA from the forearm with endothelin-1 infusion despite a 63% reduction in blood flow at the higher dose. Basal t-PA release is of the order of ~0.9 ng/100 mL of tissue/min in the forearm<sup>16</sup> and the apparent trend for an increase in t-PA antigen concentrations may, in part, reflect the reduction in blood flow associated with the marked forearm vasoconstriction (see Fig. 3). This is borne out by the unchanged t-PA activity, because it would be anticipated that plasma PAI-1 and t-PA antigen concentrations would increase proportionately with reductions in blood flow. ET<sub>B</sub> receptor antagonism causes both inhibition of endothelium-derived vasodilators such as nitric oxide, and potential hyperstimulation of the unopposed ET<sub>A</sub> receptor. However, as with endothelin-1, BQ-788 did not affect plasma concentrations of t-PA or PAI-1 in the infused forearm.

Forearm release of t-PA has been demonstrated using various endothelial cell stimulants including methacholine,<sup>16,26</sup> noradrenaline<sup>17</sup> and desmopressin.<sup>27</sup> Using the same technique as in the present study, we have previously demonstrated *in vivo* t-PA release of up to 80 ng/100 mL of tissue/min across the human forearm using intrabrachial substance P infusion<sup>14</sup> and this release is sustained for at least 2 h.<sup>15</sup> In contrast, stimulation or antagonism of the endothelial ET<sub>B</sub> receptor, with endothelin-1 and BQ-788 respectively, does not appear to influence forearm t-PA release. It is, therefore, unlikely that endothelin-1 provides a major contribution to the regulation of t-PA release in man, although we cannot exclude a small stimulatory effect.

### Study limitations

In the forearm, typical resting arterio-venous differences are only ~10% of the total venous t-PA concentration and the basal constitutive release of t-PA antigen is ~0.9 ng/100 mL of tissue/min.<sup>16</sup> We have measured venous-venous differences between the infused and non-infused arms which, unlike the measurement of arterio-venous differences of the infused arm, has the disadvantage of not being able to correct for blood-flow-dependent changes in venous plasma t-PA concentrations. Theoretically (see Fig. 3), in the absence of an

alteration in t-PA release, a 60% reduction in blood flow would be anticipated to increase total venous plasma t-PA concentrations by only ~7%, whereas a 200% increase in flow would reduce t-PA concentrations to the same degree (~7%). In the presence of stimulated t-PA release, these small flow-dependent changes are proportionately reduced even further.

The measurement of arterio-venous differences necessitates arterial sampling and the insertion of large-bore cannulae (19–20 gauge) which do not lend themselves to multiple cannulations within the same subject. Moreover, there is also the potential to introduce artefact from the presence of a larger thrombogenic surface, given that activated factor Xa is the most potent stimulant for t-PA release yet known.<sup>28</sup> To minimize arterial trauma and facilitate repeated studies in the same subjects, we have used 27-gauge arterial cannulae which permit drug infusion but not arterial blood sampling. However, we would suggest that flow-dependent changes in venous t-PA concentrations are small, within the variability of the t-PA assays (~5–7%) and are not of practical importance. Interestingly, a significant fall in the arterio-venous difference, or venous plasma concentration, of t-PA has not been detected during blood flow increases of up to 600% with sodium nitroprusside infusion.<sup>14,16,29</sup>

Measurement of venous-venous and arterio-venous differences both have the potential limitation that they can only estimate the net release of t-PA from the forearm and are unable to take account of clearance of t-PA within the forearm. However, the majority of t-PA is removed from the circulation by the liver<sup>30</sup> and the contribution of forearm clearance of t-PA is, therefore, likely to be very small.

During the present study, we did not see changes in heart rate or blood pressure to suggest systemic effects of endothelin-1<sup>31</sup> or BQ-788 infusion.<sup>32</sup> However, measuring venous concentrations bilaterally will control for any potential systemic effects which may go unrecognized if arterio-venous differences are measured in isolation. Once a drug has a systemic rather than a local effect, there is always the concern that subsequent t-PA release may be influenced or mediated by the release of other humoral factors, such as catecholamines. Moreover, if the main mechanism of t-PA release is mediated by a systemically released intermediate factor, then measuring arterio-venous differences could fail to detect this since arterial concentrations may remain unchanged and venous concentrations will rise in both forearms.

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## **PUBLICATION 4**

## Fibrinolytic actions of intra-arterial angiotensin II and bradykinin in vivo in man

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### Abstract

**Objectives:** Angiotensin II and bradykinin are potent endogenous vasoactive peptides which may play a role in the regulation of endogenous fibrinolysis and, thereby, contribute to the beneficial actions of ACE inhibitors. The aims of the study were to determine the acute effect of angiotensin II and bradykinin on the local vascular release of tissue plasminogen activator (t-PA) and its inhibitor, plasminogen activator inhibitor type 1 (PAI-1), and the endothelium derived haemostatic factor, von Willebrand factor (vWf) from the forearm. **Methods:** Blood flow, and plasma haemostatic and fibrinolytic factors, were measured in both forearms of sixteen healthy men: eight subjects received intra-arterial angiotensin II (5, 50 and 500 pmol/min) which was coinfused with sodium nitroprusside (SNP; 0.3, 1.5 and 7.5 µg/min, respectively), and eight received intra-arterial bradykinin at 10–3000 pmol/min. **Results:** Despite substantial rises in plasma angiotensin II concentrations ( $P < 0.001$ ) which caused pressor effects ( $P < 0.003$ ) at the highest dose, angiotensin II infusion did not affect local plasma t-PA, PAI-1 or vWf concentrations. In contrast, bradykinin caused substantial dose dependent increases in blood flow and t-PA release ( $> 100$  ng/100 ml of tissue/min) in the infused forearm ( $P < 0.001$  for both) without affecting plasma PAI-1 or vWf concentrations. **Conclusions:** Despite high local concentrations with breakthrough of significant systemic effects, angiotensin II did not affect acute endothelial cell t-PA, PAI-1 or vWf release in healthy men. In contrast, bradykinin is a potent vasodilator and selective stimulus for acute local t-PA release. This may, at least in part, explain the fibrinolytic actions of ACE inhibitors in heart failure and ischaemic heart disease. © 2000 Elsevier Science B.V. All rights reserved.

**Keywords:** Thrombolysis; Endothelial Factors; Blood Flow; Angiotensin

*This article is referred to in the Editorial by L. Dézsi (pages 642–644) in this issue.*

### 1. Introduction

Tissue plasminogen activator (t-PA), and its inhibitor, plasminogen activator inhibitor type 1 (PAI-1), are potentially important endothelium derived mediators that are intimately linked to the risk of thrombosis. Acute t-PA

release results from the rapid translocation of a dynamic intracellular storage pool present in the endothelium [1] of the precapillary arterioles and post capillary venules [2]. The time course of t-PA release is important since clot dissolution is much more effective if t-PA is incorporated during clot formation rather than following completion [3,4].

In patients with hypertension, an elevated plasma renin activity for a given urinary sodium excretion is independently associated with an increased risk of acute myocardial infarction [5]. Moreover, large scale clinical trials in patients with heart failure, ischaemic heart disease or a

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recent myocardial infarction suggest a reduction in reinfarction rates with angiotensin converting enzyme (ACE) inhibitor therapy. The mechanisms underlying the association of renin–angiotensin system activation with coronary thrombotic events are unknown but may relate, in part, to an effect on fibrinolytic parameters. Indeed, activation of the renin–angiotensin system by sodium depletion causes an increase in early morning plasma PAI-1 concentrations that can be reversed by ACE inhibition [6]. Furthermore, in patients with heart failure [7] or a recent myocardial infarction [8,9], ACE inhibitor therapy causes marked reductions in plasma PAI-1 concentrations.

The renin–angiotensin and kinin systems have a direct influence on the regulation of the fibrinolytic system. Cell culture studies demonstrate marked up-regulation and release of PAI-1 in response to angiotensin II administration [10,11] whilst *ex vivo* animal models have suggested that bradykinin is one of the most potent stimulants for the release of t-PA [12,13]. Studies in man have also indicated that systemic infusions of angiotensin II and bradykinin are associated with elevated levels of PAI-1 [14] and t-PA [15,16]. However, systemic drug administration of vasoactive agents can change blood pressure and regional blood flow, as well as having widespread effects on many tissues. Thus, altered systemic fibrinolytic parameters might be attributable, for instance, to changes in hepatic release and clearance of t-PA and PAI-1, and the concomitant release of other stimulatory, vasoactive and humoral mediators [15,17]. To avoid these potentially confounding systemic effects, the use of locally active intra-arterial infusion of drugs can be used to investigate the acute local release of t-PA and PAI-1 in the forearm of man [18–24].

We have previously been able to demonstrate that substance P, an endothelium-dependent vasodilator, causes a selective dose-dependent release of t-PA from the human forearm without causing significant release of PAI-1 or the endothelium-derived haemostatic factor, von Willebrand factor (vWf) [21,22]. In contrast, despite causing equivalent increases in blood flow, the potent endothelium-independent vasodilator, sodium nitroprusside, does not affect t-PA release from the forearm [19,21,24]. The aim of the present study was, therefore, to determine the local effect of intra-arterial angiotensin II and bradykinin administration on the local forearm release of t-PA, PAI-1 and vWf.

## 2. Methods

### 2.1. Subjects

Sixteen healthy male non-smokers, aged between 21 and 35 years, participated in the study which was undertaken with the approval of the local research ethics committee and in accordance with the Declaration of Helsinki. The written informed consent of each subject was obtained before entry into the study. None of the subjects received

vasoactive or non-steroidal anti-inflammatory drugs in the week before each phase of the study, and all abstained from alcohol for 24 h, and from food and caffeine-containing drinks for at least 12 h before each study. All studies were carried out in a quiet, temperature controlled room maintained at 22–24°C.

### 2.2. Drugs

Pharmaceutical grade bradykinin (Clinalfa, Läufelfingen, Switzerland), angiotensin II (Celinalfa) and sodium nitroprusside (Nipride; Roche, Welwyn Garden City, UK) were administered following dissolution in saline (0.9%: Baxter Healthcare, Thetford, UK).

### 2.3. Intra-arterial administration

The brachial artery of the non-dominant arm was cannulated with a 27-standard wire gauge steel needle (Cooper's Needle Works, Birmingham, UK) under 1% lignocaine (Xylocaine: Astra Pharmaceutical, Kings Langley, UK) local anaesthesia and attached to a 16-gauge epidural catheter (Portex, Hythe, UK). Patency was maintained by infusion of saline via an IVAC P1000 syringe pump (IVAC, Basingstoke, UK). The total rate of intra-arterial infusions was maintained constant throughout all studies at 1 ml/min.

### 2.4. Measurements

#### 2.4.1. Forearm blood flow and haemodynamics

Blood flow was measured in both forearms by venous occlusion plethysmography using mercury-in-silastic strain gauges applied to the widest part of the forearm [25]. During measurement periods, the hands were excluded from the circulation by rapid inflation of the wrist cuffs to a pressure of 220 mmHg using E20 rapid cuff inflators (D.E. Hokanson, Washington, USA). Upper arm cuffs were inflated intermittently to 40 mmHg for 10 s in every 15 s to achieve venous occlusion and obtain plethysmographic recordings. Analogue voltage output from an EC-4 strain gauge plethysmograph (D.E. Hokanson) was processed by a MacLab<sup>®</sup> analogue-to-digital converter and CHART<sup>™</sup> v3.3.8 software (AD Instruments, Castle Hill, Australia) and recorded onto a MacIntosh Classic II computer (Apple Computers, Cupertino, CA, USA). Calibration was achieved using the internal standard of the plethysmograph.

Blood pressure and heart rate were monitored in the non-infused arm at intervals throughout each study using a semi-automated non-invasive oscillometric sphygmomanometer (Takeda UA 751, Takeda Medical, Tokyo, Japan) [26].

### 2.5. Assays

Venous cannulae (17G) were inserted into large subcuta-



neous veins of the antecubital fossa in both arms. A 10–20 ml volume of blood was withdrawn simultaneously from each arm and collected into acidified buffered citrate (Biopool® Stabilyte™, Umeå, Sweden; for t-PA assays), citrate (Monovette®, Sarstedt, Nümbrecht, Germany; for PAI-1 and vWf assays) and 0.45% o-phenanthroline–4.65% disodium ethylene diamine tetraacetic acid (for angiotensin II assays) tubes, and kept on ice before being centrifuged at 2000 g for 30 min at 4°C. Platelet free plasma was decanted and stored at –80°C before assay [27].

Plasma PAI-1 and t-PA antigen concentrations were determined using an ELISA; Coaliza® PAI-1 [28] and Coaliza® t-PA [29] (Chromogenix, Mölndal, Sweden), respectively. Plasma PAI-1 and t-PA activities were determined by a photometric method, Coatest® PAI-1 [30] and Coaset® t-PA [31] (Chromogenix). Intra-assay coefficients of variation were 5.5 and 7.0% for t-PA and PAI-1 antigen, and 2.4 and 4.0% for activity, respectively. Inter-assay coefficients of variability were 4.0, 7.3, 4.0 and 7.6%, respectively. The sensitivities of the assays were 0.5 ng/ml, 2.5 ng/ml, 0.10 I.U./ml and 5 AU/ml, respectively. Determination of vWf antigen [32] was undertaken using an ELISA (Dako, Glostrup, Denmark) with a sensitivity of 0.05 I.U./ml. The intra-assay and inter-assay coefficients of variability were 5.2 and 7.3%, respectively. Following extraction using Bond Elut® columns (Varian, Harbor City, CA, USA), plasma angiotensin II (Peninsula Labs. Europe, St. Helens, UK) concentrations were determined by radioimmunoassay as previously described [33]. The intra- and inter-assay coefficients of variability were 7.2 and 9.3%, respectively. Haematocrit was determined by capillary tube centrifugation of blood anticoagulated by ethylene diamine tetraacetic acid and was obtained from the infused forearm at baseline and at the end of the study protocol.

## 2.6. Study design

Subjects rested recumbent, and strain gauges and cuffs were applied. The brachial artery of the non-dominant arm was cannulated and forearm blood flow measured every 10 min or between 3 and 6 min for shorter (6 min) infusion periods during the bradykinin protocol. Before bradykinin or combined angiotensin II and sodium nitroprusside administration, saline was infused for 30 min to allow time for equilibration. The final blood flow measurement during saline infusion was taken as the basal forearm blood flow.

### 2.6.1. Angiotensin II protocol

There is a modest basal release of t-PA from the forearm and, in the presence of a reduction in blood flow, an apparent rise in t-PA concentrations may occur without a change in the rate of t-PA release [34]. To correct for any flow dependent effects, sodium nitroprusside, which does not cause t-PA release [19,21,24], was coinfused with angiotensin II to offset the angiotensin II mediated vas-

oconstriction and maintain forearm blood flow greater than or equal to that during saline infusion. Eight subjects received three incremental infusions of angiotensin II and sodium nitroprusside for 30 min at each dose in the following order: angiotensin II 5 pmol/min+sodium nitroprusside 0.3 µg/min; angiotensin II 50 pmol/min+sodium nitroprusside 1.5 µg/min; and angiotensin II 500 pmol/min+sodium nitroprusside 7.5 µg/min. The doses of sodium nitroprusside were determined from preliminary pilot studies (data on file). Venous blood samples were obtained from both forearms at baseline and every 10 min thereafter.

### 2.6.2. Bradykinin protocol

Eight subjects received an incremental infusion of bradykinin at 10, 30, 100, 300, 1000 and 3000 pmol/min followed by a 20-min saline infusion. Venous blood samples were obtained from both forearms at baseline, during bradykinin infusion at 30, 300 and 3000 pmol/min, and 10 and 20 min after cessation of the final bradykinin infusion of 3000 pmol/min. Bradykinin was infused for 6 min at each dose but was extended to 10 min at the 30, 300 and 3000 pmol/min doses because of venous sampling following blood flow measurements.

## 2.7. Data analysis and statistics

The study population size, based on power calculations derived from previous studies [21], gives 90% power of detecting a 21% difference in t-PA release at a significance level of 5%. Coefficients of repeatability [35] for plasma concentrations of t-PA antigen and activity during substance P infusion at 8 pmol/min are 1.6 ng/ml and 1.4 I.U./ml, respectively (data on file).

Plethysmographic data were extracted from the CHART data files. Forearm blood flows were calculated for individual venous occlusion cuff inflations by use of a template spreadsheet (EXCEL v5.0; Microsoft, Cambridge, USA). Recordings from the first 60 s after wrist cuff inflation were not used because of the variations in blood flow that this causes. Usually, the last five flow recordings in each 3-min measurement period were calculated and averaged for each arm. Estimated net release of t-PA activity and antigen was defined previously [21] as the product of the infused forearm plasma flow (based on the mean haematocrit, Hct, and the infused forearm blood flow, FBF) and the concentration difference between the infused ([t-PA]<sub>inf</sub>) and non-infused arms ([t-PA]<sub>non-inf</sub>)

$$\text{Estimated net t-PA release} = \text{FBF} \times \{1 - \text{Hct}\} \\ \times \{[\text{t-PA}]_{\text{inf}} - [\text{t-PA}]_{\text{non-inf}}\}$$

Data were examined, where appropriate, by multifactorial analysis of variance (ANOVA) with repeated measures and two-tailed paired Student's *t*-test using EXCEL v5.0 (Microsoft). All results are expressed as mean±standard error

Table 1  
Subject characteristics

		Angiotensin II protocol	Bradykinin protocol
Age	(years)	26±2	25±2
Height	(m)	1.85±0.02	1.78±0.03
Weight	(kg)	81±2	81±4
Body mass index	(kg/m <sup>2</sup> )	23±1	25±1
Body surface area	(m <sup>2</sup> )	2.03±0.04	1.99±0.06
Heart rate	(/min)	63±3	68±6
Blood pressure	Systolic (mmHg)	132±5	132±7
	Diastolic (mmHg)	74±2	78±4
Haematocrit	Basal	0.38±0.00	0.40±0.02
	Final	0.38±0.01	0.41±0.01

of the mean. Statistical significance was taken at the 5% level.

### 3. Results

Subject characteristics are shown in Table 1.

#### 3.1. Angiotensin II protocol

There were no significant changes in blood pressure,

heart rate or blood flow in the non-infused arm throughout the study protocol except during the final 30 min infusion (Fig. 1).

In comparison with the preceding 30-min infusion periods, administration of angiotensin II 500 pmol/min with sodium nitroprusside 7.5 µg/min was associated with an increase in mean arterial pressure (four-way ANOVA,  $P<0.003$ ), and infused (four-way ANOVA,  $P<0.001$ ) and non-infused (four-way ANOVA,  $P=0.05$ ) forearm blood flow without a change in heart rate.

Venous plasma angiotensin II concentrations increased in the infused (ANOVA,  $P<0.001$  for all doses) and non-infused (ANOVA,  $P<0.001$  at 500 pmol/min only) forearms (Table 2). In comparison to the non-infused arm, plasma angiotensin II concentrations were substantially greater in the infused arm (Table 2; two-way ANOVA,  $P<0.001$ ). However, despite substantial increases in local angiotensin II concentrations, there were no significant changes in plasma t-PA, PAI-1 or vWf antigen concentrations in either arm (Table 2).

#### 3.1.1. Bradykinin protocol

Consistent with the actions of other kinins [21], transient and patchy flushing and skin oedema of the infused arm occurred with bradykinin infusion at doses  $\geq 300$  pmol/

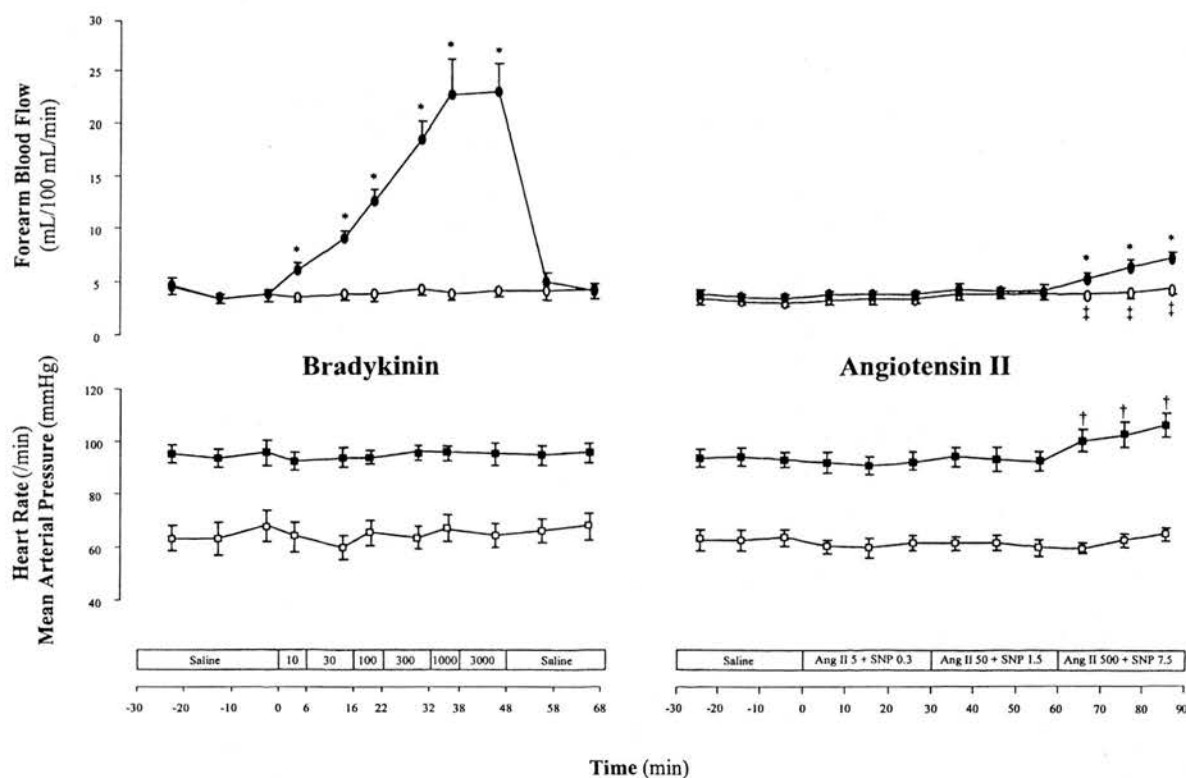


Fig. 1. Mean arterial pressure (solid squares), heart rate (open squares), and blood flow in the infused (solid circles) and non-infused (open circles) forearms during saline, bradykinin (pmol/min), and combined angiotensin II (Ang II, pmol/min) and sodium nitroprusside (SNP, µg/min) infusions. Paired *t*-test: \*,  $P<0.001$ , †,  $P\leq 0.02$ , ‡,  $P\leq 0.05$  (vs. baseline); One-way ANOVA:  $P<0.001$  for infused forearm blood flow (bradykinin and angiotensin II infusions). Two-way ANOVA:  $P<0.001$  (infused vs. non-infused forearm blood flow, bradykinin and angiotensin II infusions); †,  $P=0.006$ , ‡,  $P=0.02$  (saline vs. angiotensin II 500 pmol/min); four-way ANOVA: †,  $P<0.003$ , ‡,  $P=0.05$  (four groups: saline, angiotensin II 5 pmol/min, angiotensin II 50 pmol/min and angiotensin II 500 pmol/min).

Table 2

Estimated net release and plasma concentrations of tissue plasminogen activator (t-PA) and plasminogen activator inhibitor type 1 (PAI-1) antigen, and venous plasma von Willebrand factor (vWf) and angiotensin II concentrations during angiotensin II and sodium nitroprusside infusion

	Baseline	Angiotensin II 5 pmol/min + sodium nitroprusside 0.3 µg/min			Angiotensin II 50 pmol/min + sodium nitroprusside 1.5 µg/min			Angiotensin II 500 pmol/min + sodium nitroprusside 7.5 µg/min		
Time (min)	0	10	20	30	40	50	60	70	80	90
t-PA antigen (ng/ml)										
Infused arm	3.4±0.4	3.7±0.4	3.6±0.3	3.4±0.4	3.4±0.4	3.6±0.3	3.5±0.4	3.4±0.2	3.4±0.3	3.5±0.3
Non-infused arm	3.7±0.5	3.4±0.3	3.3±0.4	3.6±0.4	3.4±0.4	3.4±0.3	3.3±0.3	3.5±0.3	3.4±0.2	3.3±0.3
Concentration difference between forearms	-0.3±0.1	0.3±0.2	0.3±0.1	-0.2±0.1	0.0±0.1	0.2±0.1	0.2±0.1	-0.1±0.1	0.0±0.1	0.1±0.1
Estimated net release of t-PA (ng/100 ml/min)	-0.4±0.2	0.4±0.2	0.4±0.2	-0.2±0.1	-0.1±0.2	0.3±0.1	0.3±0.2	-0.1±0.2	0.1±0.2	0.3±0.3
PAI-1 antigen (ng/ml)										
Infused arm	16±4	16±5	15±4	15±3	12±3	13±4	13±3	11±3	13±3	12±3
Non-infused arm	14±4	15±4	14±4	14±4	13±3	12±4	13±3	12±3	11±3	11±3
Concentration difference between forearms	2±1	2±2	1±1	1±1	0±0	1±1	0±2	0±1	1±1	1±1
Estimated net release of PAI-1 (ng/100 ml/min)	3±2	2±2	0±1	0±1	-1±1	2±2	0±3	-1±2	2±1	3±3
vWf Antigen (I.U./ml)										
Infused arm	0.7±0.1	0.6±0.1	0.6±0.1	0.7±0.1	0.7±0.1	0.7±0.1	0.7±0.1	0.8±0.2	0.8±0.2	0.7±0.1
Non-infused arm	0.8±0.2	0.8±0.2	0.7±0.2	0.7±0.2	0.6±0.1	0.8±0.2	0.8±0.2	0.8±0.2	0.9±0.1	0.9±0.2
Plasma angiotensin II concentration (fmol/ml)*										
Infused arm	8±3	—	92±11‡	—	—	174±3‡	—	—	219±23‡	-†
Non-infused arm	12±5	—	11±4	—	—	46±26	—	—	126±19‡	-†

\* n=4.

One-way ANOVA: †,  $P \leq 0.001$ . Two-way ANOVA: ‡,  $P < 0.001$  (infused vs. non-infused forearm). Paired *t*-test: ‡,  $P \leq 0.01$  (vs. baseline).

min. The oedema had an urticarial appearance, taking the form of a raised wheal with a yellow hue. The extent of oedema varied between subjects, beginning at the level of the elbow and extending distally with increasing dose. However, there was no associated pruritis or tenderness and the oedema resolved completely within 1–2 h of stopping the infusion. No local effects were seen with angiotensin II and sodium nitroprusside infusions.

There were no significant changes in blood pressure, heart rate or blood flow in the non-infused arm throughout the study protocol (Fig. 1). Bradykinin increased blood flow in the infused arm (ANOVA,  $P < 0.001$ ) in a dose dependent manner (Fig. 1) reaching a maximum increase of  $22.8 \pm 2.7$  ml/100 ml/min at 3000 pmol/min.

Bradykinin caused a dose-dependent increase in venous plasma t-PA activity and antigen concentrations of the infused (ANOVA,  $P < 0.001$ ) and non-infused (ANOVA,  $P = 0.01$ ) forearms (Fig. 2). In comparison to the non-infused arm, plasma t-PA antigen and activity concentrations were substantially greater in the infused arm (two-way ANOVA,  $P < 0.001$ ), consistent with a marked net local release of t-PA (ANOVA,  $P < 0.001$ ; Table 3).

Baseline plasma PAI-1 antigen, PAI-1 activity and vWf concentrations were  $36 \pm 4$  ng/ml,  $12 \pm 2$  and  $0.64 \pm 0.05$  I.U./ml in the infused arm, and  $40 \pm 4$  ng/ml,  $13 \pm 1$  and  $0.67 \pm 0.09$  I.U./ml in the non-infused arm, respectively. There were no significant changes in plasma PAI-1 antigen, PAI-1 activity or vWf concentrations in either arm during bradykinin infusion (data on file).

#### 4. Discussion

We have shown that, despite very high local concentrations, ultimately causing significant systemic pressor effects, angiotensin II infusion does not directly affect acute local endothelial cell t-PA or PAI-1 release. In contrast, we found that the potent vasodilator, bradykinin, is a selective stimulus for acute local t-PA release. These findings suggest that bradykinin, but not angiotensin II, may contribute to the acute local regulation of endogenous fibrinolysis in man. This may, in part, contribute to altered endogenous fibrinolysis seen with the use of ACE inhibitor therapy [8,9].

In vitro studies have suggested that angiotensin II influences endothelial [11] and vascular smooth muscle cell [10] PAI-1 synthesis and release. However, in vitro cell techniques have limitations because the phenotype of cells in culture, and the ability to release PAI-1, may change, particularly with increasing passages. There has been conflicting in vivo evidence with regard to the role of angiotensin II in the regulation of endogenous fibrinolysis, with angiotensin II either increasing plasma PAI-1 concentrations without affecting t-PA [14] or increasing plasma t-PA concentrations without affecting PAI-1 [16]. However, systemic neurohumoral responses to the infusion of vasoactive agents may contribute to, or even mediate, the subsequent fibrinolytic response [17]. To avoid these confounding systemic effects, locally active intra-arterial infusion of drugs can be used to investigate the acute local

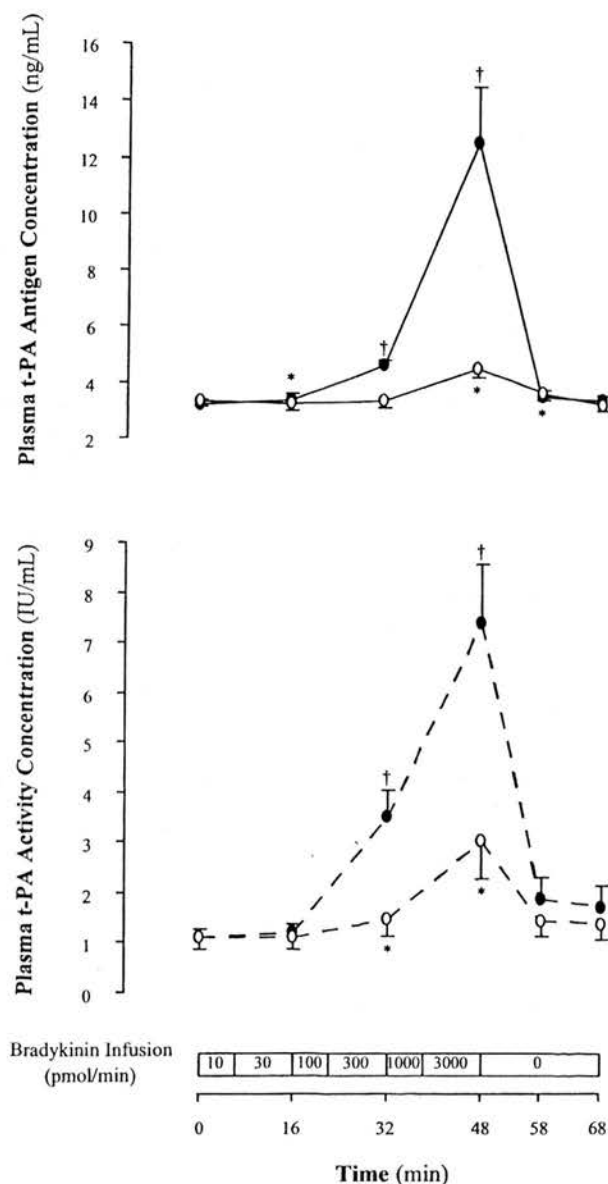


Fig. 2. Plasma tissue plasminogen activator (t-PA) antigen (solid lines; upper panel) and activity (dashed lines; lower panel) concentrations in the infused (solid circles) and non-infused (open circles) forearms during saline and bradykinin infusions. One-way ANOVA: infused arm;  $P < 0.001$  for t-PA antigen and activity. Non-infused arm;  $P = 0.003$  for t-PA antigen,  $P = 0.01$  for t-PA activity. Two-way ANOVA: infused vs. non-infused arm;  $P < 0.001$  for t-PA antigen and activity. Paired  $t$ -test: \*,  $P < 0.05$ , †,  $P < 0.001$  (vs baseline).

release of t-PA in the forearm circulation of man [18–24]. Using high but locally vasoactive doses of angiotensin II (5 and 50 pmol/min), we were unable to detect alterations in plasma t-PA or PAI-1 concentrations in the forearm vascular bed. Moreover, the absence of a fibrinolytic response is unlikely to be a temporal effect since the blood vessels of the infused forearm were exposed to markedly elevated plasma angiotensin II concentrations for 90 min.

In order to counteract the development of forearm vasoconstriction and subsequent flow-dependent changes

in plasma t-PA concentrations [34], sodium nitroprusside was co-infused with angiotensin II. Sodium nitroprusside is unlikely to have influenced the fibrinolytic response to angiotensin II infusion because a number of previous *in vitro* [36] and *in vivo* studies [19–21,24] have failed to demonstrate any effect of sodium nitroprusside administration on the acute release of t-PA or PAI-1. At the highest dose, angiotensin II did not affect plasma t-PA or PAI-1 concentrations despite increasing systemic blood pressure and increasing forearm blood flow in the non-infused arm. Increased blood flow of the infused forearm may, at least in part, reflect relative dominance of the vasodilatation associated with sodium nitroprusside co-infusion. However, vasodilatation of the non-infused forearm with systemic pressor doses of angiotensin II is consistent with previous work [37] and is related to the relatively greater vasoconstrictor response to angiotensin II infusion in other resistance vascular beds, such as the splanchnic circulation.

In addition to potential actions on plasma fibrinogen [38] and platelet function [39], the major benefits associated with ACE inhibitor therapy may, at least in part, be mediated through actions on endogenous fibrinolytic function. Following myocardial infarction, basal fibrinolytic parameters are improved by the use of ACE inhibitors [8,9], principally through a reduction in plasma PAI-1 concentrations. The absence of a direct effect of high local concentrations of angiotensin II in the forearm circulation suggests that this is not mediated by a direct action of angiotensin II on the endothelium. However, PAI-1 is synthesised and released by many tissues, including megakaryocytes, liver and vascular smooth muscle, and the present study does not exclude a systemic role of angiotensin II in the regulation of endogenous fibrinolysis.

Animal models suggest that bradykinin is an extremely potent stimulus for the release of t-PA from the vascular endothelium *in vitro* [1], *ex vivo* [13] and *in vivo* [40]. We have been able to demonstrate marked and substantial release of t-PA with intra-arterial infusions of bradykinin in humans. Assuming a forearm volume of ~1000 ml [25], 3000 pmol/min of bradykinin caused the release of over 1  $\mu$ g/min of t-PA from the infused forearm. Plasma t-PA concentrations, but not blood flow, increased in the non-infused forearm at the highest dose of bradykinin, suggesting the release of t-PA from the infused forearm exceeded the capacity to clear it immediately from the systemic circulation.

Recently, Brown et al. [41] have described the forearm release of t-PA with intra-arterial bradykinin infusion using doses up to 400 pmol/min. Using similar techniques, we have confirmed their findings, shown a greater release of t-PA at higher doses of bradykinin, and extended these observations to demonstrate the absence of vWf release. In contrast to the animal studies [13], bradykinin does not appear to release vWf from the forearm circulation in man. This finding is consistent with similar observations obtained with substance P [21] and desmopressin infusions [20], and suggests the presence of separate and distinct



Table 3

Estimated net release of tissue plasminogen activator (t-PA) and plasminogen activator inhibitor type 1 (PAI-1) antigen and activity during bradykinin infusion

	Bradykinin (pmol/min) Time (min)	Baseline	Bradykinin				Saline	
		0	30	300	3000		0	0
		0	16	32	48		58	68
t-PA antigen	Concentration difference between forearms (ng/ml)	-0.2±0.1	0.1±0.1	1.3±0.1‡	8.1±1.75‡		-0.1±0.3	0.2±0.1*
	Net local (infused) forearm release (ng/100 ml/min)	-0.4±0.2	0.8±0.8‡	14.0±1.6‡	107.5±27.6‡		-0.5±0.9	0.4±0.2*
t-PA activity	Concentration difference between forearms (I.U./ml)	-0.0±0.1	0.1±0.1	2.0±0.4‡	4.4±1.0‡		0.5±0.2	0.3±0.1*
	Net local (infused) forearm release (I.U./100 ml/min)	0.1±0.2	0.5±0.4	21.1±3.2‡	57.5±14.2‡		0.9±0.3	0.7±0.2*
PAI-1 antigen	Concentration difference between forearms (ng/ml)	-3±3	-4±2	-6±6	-3±4		0±3	-5±3
	Net local (infused) forearm release (ng/100 ml/min)	-16±10	-23±10	-61±66	-30±56		1±9	-9±6
PAI-1 activity	Concentration difference between forearms (AU/ml)	-1±2	0±1	-1±2	-1±2		2±2	2±3
	Net local (infused) forearm release (AU/100 ml/min)	1±11	-3±7	-12±24	-14±23		7±5	3±7

One-way ANOVA: \*,  $P < 0.001$ .

Paired *t*-test: †,  $P < 0.05$ , ‡,  $P \leq 0.001$  (vs. baseline).

endothelial storage pools and release mechanisms for t-PA and vWf.

Small areas of denudation and thrombus deposition are a common finding on the surface of atheromatous plaques [42,43] and are usually sub-clinical. However, in the presence of an imbalance in the coagulation or fibrinolytic systems, such microthrombi may propagate, ultimately leading to arterial occlusion. Bradykinin is not only an inflammatory mediator but is also released during the contact phase of coagulation when high-molecular-weight kininogen is cleaved by kallikrein to produce a disulphide-linked light and heavy chain [44,45]. This liberation of bradykinin may represent an important negative feedback loop in which bradykinin induced t-PA release inhibits thrombus formation within the vascular lumen when localised endothelial denudation occurs. Furthermore, given that bradykinin induced forearm vasodilatation is potentiated by ACE inhibition [46], such actions may be enhanced in the presence of ACE inhibition and may, in part, explain the anti-ischaemic action of this therapy [47]. However, although inferred, the potentiation of bradykinin induced t-PA release by ACE inhibition has yet to be established.

In conclusion, it would appear that bradykinin, but not angiotensin II, is a potent vasodilator and selective stimulus for acute local t-PA release. This may, in part, explain the beneficial fibrinolytic actions of ACE inhibition in heart failure and ischaemic heart disease.

## Acknowledgements

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## **PUBLICATION 5**

# Bradykinin Receptor Antagonism and Endothelial Tissue Plasminogen Activator Release in Humans

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**Objective**—We sought to assess pharmacodynamic responses to the bradykinin antagonist B9340 and to determine the contribution of the endothelial bradykinin receptor to stimulated tissue plasminogen activator (t-PA) release in humans.

**Methods and Results**—Bilateral forearm blood flow and plasma fibrinolytic variables were measured in 8 volunteers during 100 minutes of intrabrachial infusions of saline placebo, B9340 at 4.5 nmol/min, or B9340 at 13.5 nmol/min. On each occasion, intra-arterial bradykinin (30 to 3000 pmol/min) and substance P (4 to 16 pmol/min) were coinfused for 10 minutes at each dose. To assess the onset and offset of action, 6 additional subjects on 2 occasions received intra-arterial bradykinin (100 pmol/min) for 60 minutes with a coinfusion of either saline placebo or B9340 (13.5 nmol/min) for 12 minutes. During placebo infusion, bradykinin and substance P caused dose-dependent vasodilatation in the infused forearm ( $P < 0.001$ ). B9340 caused a dose-dependent inhibition of bradykinin-induced forearm vasodilatation and t-PA release ( $P < 0.001$ ) without affecting substance P-induced vasodilatation or t-PA release ( $P = \text{NS}$ ). B9340 caused a reversible inhibition of bradykinin-induced vasodilatation ( $P < 0.001$ ) with a rapid onset and offset of action.

**Conclusions**—B9340 is a potent, reversible, and selective competitive receptor antagonist of bradykinin-induced vasodilatation and t-PA release in humans. (*Arterioscler Thromb Vasc Biol.* 2003;23:1667-1670.)

**Key Words:** bradykinin ■ blood flow ■ tissue plasminogen activator ■ receptor antagonism

Bradykinin is an endogenous, vasoactive, nonapeptide mediator involved in many physiologic processes. It is cleaved from high-molecular-weight kininogen during the contact phase of blood coagulation,<sup>1</sup> resulting in endothelium-dependent vasodilatation and stimulation of tissue plasminogen activator (t-PA) release from human endothelial cells.<sup>2</sup> It has a brief duration of action (plasma half-life of 15 to 30 seconds) owing to its rapid degradation by several enzymes, principally angiotensin-converting enzyme (ACE). Bradykinin appears to contribute to the vascular effects of ACE inhibitor therapy in hypertension<sup>3</sup> and heart failure.<sup>4</sup>

Bradykinin receptor antagonists have been developed from peptide analogues of bradykinin. The most widely used bradykinin receptor antagonist is HOE-140, or icatibant, which demonstrates high selectivity for the B<sub>2</sub> receptor. Recently, a third-generation synthetic peptide antagonist of bradykinin, B9340, has been synthesized. It has a similar chemical structure to HOE-140 and differs by replacement of the  $\alpha$ -(2-indanyl)glycine at position 7 of the molecule with a tetrahydroisoquinoline-3-carboxylic acid moiety. In comparison with HOE-140, B9340 retains similar potency of inhibition at the bradykinin B<sub>2</sub> receptor (median inhibitory concen-

tration [IC<sub>50</sub>] of 0.158 nmol/L for both) but has greater inhibition at the B<sub>1</sub> receptor (IC<sub>50</sub> of 1000 nmol/L and 7.9 nmol/L for HOE-140 and B9340, respectively).<sup>5</sup>

The purpose of this study was to assess pharmacodynamic responses to the novel bradykinin antagonist B9340 and to determine the contribution of the endothelial bradykinin receptor to stimulated t-PA release in vivo in humans. To assess the selectivity of B9340, we compared the vasomotor and fibrinolytic responses of bradykinin with those of substance P, a bradykinin receptor-independent, endothelium-dependent vasodilator and stimulator of t-PA release.<sup>6</sup>

## Methods

### Study Subjects

Fourteen healthy, male volunteers were recruited into the study, which was performed with approval of the local ethics committee, in accordance with the Declaration of Helsinki and the written, informed consent of each subject. Each volunteer was studied at the same time of day and had been fasting for at least 4 hours before each study. All subjects avoided alcohol for 24 hours and caffeine-containing products for 5 hours before study. No medications or vasoactive drugs were taken in the 7 days before each study.

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### Intra-Arterial Administration

All studies were performed in a quiet, temperature-controlled (22°C to 25°C) room. On each occasion, the brachial artery of the nondominant arm was cannulated with a 27-gauge needle (Cooper's Needle Works Ltd) after 1% lidocaine local anesthesia. The needle was attached to a 16-gauge epidural catheter (Portex Ltd), and needle patency was maintained by an infusion of 0.9% saline at 1 mL/min. The total rate of intra-arterial infusions was maintained constant throughout all studies at 1 mL/min.

### FBF and Blood Pressure

Forearm blood flow (FBF) was measured by mercury-in-silicone elastomer strain-gauge venous-occlusion plethysmography, as previously described.<sup>7,8</sup> Immediately after each FBF measurement, pulse and blood pressure were measured noninvasively in the noninfused arm throughout each study with a semiautomated digital sphygmomanometer (UA-731, A&D Engineering). Mean arterial pressure was defined as the diastolic blood pressure plus one third of the pulse pressure.

### Drugs

Pharmaceutical-grade B9340 (Clinalfa AG), substance P (Clinalfa), and bradykinin (Clinalfa) were dissolved in 0.9% saline before infusion. All solutions were freshly prepared on the day of study.

### Venous Sampling and Assays

Venous cannulas (17 gauge) were inserted into large subcutaneous veins of the antecubital fossa in both arms, as described previously.<sup>9</sup> Ten milliliters of blood was withdrawn simultaneously from each arm and collected into acidified, buffered citrate (Biopool Stabilyte) and citrate (Monovette, Sarstedt) tubes and kept on ice before being centrifuged at 2000g for 30 minutes at 4°C. Platelet-free plasma was decanted and stored at -80°C before assay. Plasma t-PA antigen and activity concentrations were determined by ELISA (Coalza t-PA, Chromogenix AB) and a photometric method (Coatest t-PA, Chromogenix AB), respectively, as described previously.<sup>10</sup>

### Study Design

#### Protocol 1: Efficacy and Selectivity of B9340

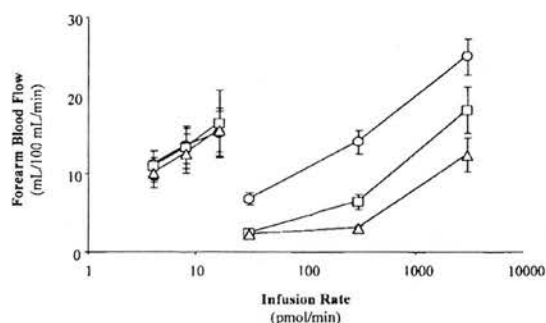
Eight healthy, male volunteers attended on 3 occasions at least 1 week apart. After 30 minutes' equilibration with saline infusion, intra-arterial placebo (0.9% saline), B9340 at 4.5 nmol/min, or B9340 at 13.5 nmol/min was infused for 100 minutes on separate occasions in a randomized, double-blind, ascending-dose design. Placebo/B9340 was coinfused with saline for 10 minutes; bradykinin at 30, 300, and 3000 pmol/min for 10 minutes at each dose; and after a 30-minute saline infusion, substance P at 4, 8, and 16 pmol/min for 10 minutes at each dose. FBF and mean arterial pressure were measured every 10 minutes for the duration of the study. Venous samples were obtained at baseline, after 10 minutes of B9340/placebo infusion, and after each dose of bradykinin and substance P.

#### Protocol 2: Onset and Offset of B9340 Action

Six additional healthy, male volunteers attended on 2 occasions at least 1 week apart. After 30 minutes' equilibration with saline infusion, intrabrachial bradykinin was infused at 100 pmol/min for 60 minutes. After 12 minutes of bradykinin infusion, B9340 (Clinalfa) at 13.5 nmol/min or placebo (saline vehicle) was coinfused for 12 minutes in a randomized, double-blind manner. FBF and mean arterial pressure were measured every 6 minutes for the duration of the study.

### Data Analysis and Statistics

Plethysmographic data were extracted from the software (Chart) data files, and FBF was calculated for individual venous-occlusion cuff inflations by use of a template spreadsheet (Excel 97, Microsoft). The last 5 flow recordings in each 3-minute measurement period were calculated and averaged for each arm. The effective dose causing a 100% increase in FBF (ED<sub>100</sub>) was calculated to assess the



**Figure 1.** Infused FBF during substance P (left) and bradykinin (right) infusion with saline placebo (circles), B9340 at 4.5 nmol/min (squares), and B9340 at 13.5 nmol/min (triangles).  $P < 0.001$ , ANOVA, for all blood flow responses;  $P < 0.001$ , ANOVA, for comparisons between saline placebo and B9340 administration for bradykinin infusion (right) only.

degree of bradykinin antagonism. The percentage increase in blood flow was determined from the ratio of the FBFs, as described previously.<sup>8</sup>

Estimated net release of t-PA antigen and activity was defined as the product of the infused forearm plasma flow (based on the hematocrit [Hct]) and the infused FBF and the concentration difference between the infused ( $[t-PA]_{inf}$ ) and noninfused forearms ( $[t-PA]_{noninf}$ ): estimated net t-PA release =  $FBF \times (1 - Hct) \times ([t-PA]_{inf} - [t-PA]_{noninf})$ .

Data were examined, where appropriate, by ANOVA with repeated measures and 2-tailed, paired Student's *t* test with commercially available software (Excel 97). All results are expressed as mean  $\pm$  SEM. Statistical significance was taken at the 5% level.

### Results

The infusions were well tolerated with no major adverse events. There were no significant changes in FBF in the noninfused arm, heart rate, or blood pressure during the infusions (data not shown).

#### Protocol 1: Efficacy and Selectivity of B9340

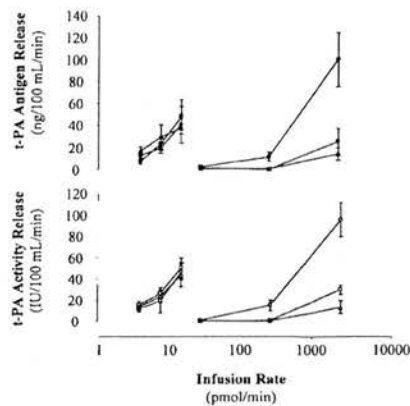
Subjects were aged  $25 \pm 5$  years with a body mass index of  $22 \pm 2$  kg/m<sup>2</sup>.

#### Forearm Blood Flow

Baseline blood flow during saline infusion was  $2.0 \pm 0.2$  mL  $\cdot$  100 mL<sup>-1</sup>  $\cdot$  min<sup>-1</sup> in the infused arm and  $2.0 \pm 0.1$  mL  $\cdot$  100 mL<sup>-1</sup>  $\cdot$  min<sup>-1</sup> in the noninfused arm. In comparison with placebo, B9340 infusion alone caused no significant change in the infused FBF from baseline at either dose (placebo,  $-7.4 \pm 3.7\%$ ; B9340 at 4.5 nmol/min,  $8.1 \pm 7.6\%$ ; and B9340 at 13.5 nmol/L,  $3.8 \pm 5.5\%$ ; ANOVA,  $P = 0.8$ ). During placebo infusion, bradykinin and substance P caused dose-dependent vasodilatation in the infused forearm (ANOVA,  $P < 0.001$  for both; Figure 1). At doses of 4.5 and 13.5 nmol/min, B9340 caused 7-fold and 18-fold increases in the ED<sub>100</sub> for bradykinin, respectively (ANOVA,  $P < 0.001$  for both; Figure 1). In contrast, B9340 had no effect on substance P-induced vasodilatation (Figure 1).

#### Estimated Net t-PA Release

Baseline plasma t-PA antigen and activity concentrations during saline infusion were  $3.4 \pm 0.4$  ng/mL and  $1.3 \pm 0.3$  IU/mL in the infused arm and  $3.6 \pm 0.3$  ng/mL and  $1.3 \pm 0.3$  IU/mL in the noninfused arm, respectively. B9340 infusion



**Figure 2.** Estimated net t-PA antigen (closed symbols, top) and activity (open symbols, bottom) release during substance P (left) and bradykinin (right) infusion with saline placebo (circles), B9340 at 4.5 nmol/min (squares), and B9340 at 13.5 nmol/min (triangles).  $P < 0.05$ , ANOVA, for all t-PA responses;  $P < 0.001$ , ANOVA, for comparisons between saline placebo and B9340 administration for bradykinin infusion (right) only.

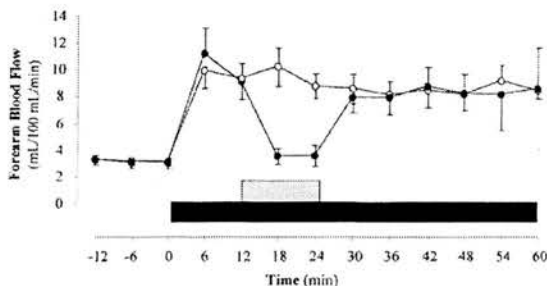
alone produced no change in plasma t-PA antigen or activity (ANOVA,  $P = 0.9$ ). Both bradykinin and substance P produced a dose-dependent increase in plasma t-PA antigen and activity concentrations in the infused forearm (ANOVA,  $P < 0.001$ ). Both doses of B9340 completely inhibited t-PA antigen and activity release at 300 pmol/min bradykinin and reduced t-PA antigen and activity release at 3000 pmol/min by 4- to 8-fold (ANOVA,  $P < 0.001$  for both doses; Figure 2). There was no effect of B9340 on substance P-induced t-PA release (Figure 2).

#### Protocol 2: Onset and Offset of B9340 Action

Subjects were aged  $30 \pm 2$  years with a body mass index of  $21 \pm 2$  kg/m<sup>2</sup>. Baseline blood flow during saline infusion was  $3.4 \pm 1.0$  mL  $\cdot$  100 mL<sup>-1</sup>  $\cdot$  min<sup>-1</sup> in the infused arm and  $2.7 \pm 0.6$  mL  $\cdot$  100 mL<sup>-1</sup>  $\cdot$  min<sup>-1</sup> in the noninfused arm. Bradykinin infusion caused a  $288 \pm 7\%$  increase in the infused FBF that was sustained for 60 minutes (ANOVA,  $P < 0.001$ ; Figure 3). Compared with placebo, B9340 coinfusion caused a rapid onset and offset of inhibition of bradykinin-induced vasodilatation (ANOVA,  $P < 0.001$ ; Figure 3).

#### Discussion

We have confirmed our earlier findings<sup>11,12</sup> that intrabrachial bradykinin infusion causes marked forearm vasodilatation



**Figure 3.** Infused-arm FBF with bradykinin infusion (black bar; 100 pmol/min) during placebo (open circles) or B9340 (closed circles; 13.5 nmol/min) coadministration (gray bar).

and endothelial t-PA release. We have demonstrated that B9340 is a potent and competitive antagonist of bradykinin-induced vasodilatation and endothelial t-PA release in vivo in humans. B9340 appears to be a selective and reversible bradykinin receptor antagonist with a rapid onset and offset of action.

B9340 caused dose-dependent inhibition of bradykinin-induced vasodilatation and t-PA release, suggesting that it acts as a competitive receptor antagonist and that both vascular effects are mediated through bradykinin receptors. Previous clinical studies<sup>13,14</sup> have used systemic intravenous administration of the selective B<sub>2</sub> receptor antagonist HOE-140, combined with intrabrachial bradykinin infusions. However, systemic drug administration might have ancillary effects, and the antagonist effects of HOE-140 could have been mediated through intermediary pathways. In contrast, here we have demonstrated a direct, local, dose-dependent inhibition of the vascular actions of bradykinin with intra-arterial infusions of B9340. Moreover, those previous studies of systemic HOE-140 administration either lacked a control vasodilator<sup>14</sup> or used the endothelium-independent vasodilator sodium nitroprusside.<sup>13</sup> We have more rigorously demonstrated the selectivity of bradykinin antagonism by B9340 through comparison with the tachykinin substance P, which acts through the endothelial neurokinin type 1 receptor to cause endothelium-dependent vasodilatation<sup>6</sup> and t-PA release. The antagonist action of B9340 cannot, therefore, be attributed to a nonspecific effect on the vascular endothelium and appears to be selective for bradykinin receptors.

Bradykinin is thought to exert its vasodilatory effects by activating the B<sub>2</sub> receptor on vascular endothelium. This results in release of nitric oxide<sup>15,16</sup> and endothelium-derived hyperpolarizing factor to produce vasorelaxation.<sup>17,18</sup> However, although inhibition of nitric oxide synthase with N<sup>G</sup>-monomethyl-L-arginine attenuates the vasodilatation to bradykinin, it does not affect endothelial t-PA release.<sup>13</sup> This would suggest that bradykinin-stimulated t-PA release is mediated through a nitric oxide synthase-independent pathway and that the endothelium regulates blood flow and t-PA release through distinct pathways. Indeed, we have recently demonstrated that direct, local, endothelial t-PA release can be induced by tumor necrosis factor- $\alpha$  in the absence of alterations in blood flow in the human forearm.<sup>19</sup> Thus, bradykinin-induced t-PA release appears to be dependent on the endothelial bradykinin receptors and to act through a second-messenger pathway that is distinct from the regulation of vasomotion.

B9340 had no effect on basal FBF or plasma t-PA concentrations, suggesting that in healthy humans, bradykinin does not contribute to the basal maintenance of vascular tone or t-PA release. However, this does not preclude a potential role for bradykinin in pathophysiologic conditions, such as inflammation,<sup>20</sup> or instances where ACE inhibitor therapy is used.<sup>4,21</sup>

Although B9340 antagonizes the B<sub>2</sub> receptor at concentrations nearly 2 orders of magnitude greater than those at the B<sub>1</sub> receptor, B9340 might still produce some blockade of the B<sub>1</sub> receptor. The B<sub>1</sub> receptor was previously thought to be inactive in the vascular system unless upregulated during

inflammation. Raidoo et al<sup>20</sup> have recently shown that B<sub>1</sub> receptors are present in large numbers on the vascular endothelium and are further upregulated around atherosclerotic plaques. However, the functional significance of the B<sub>1</sub> receptor on the endothelium is unknown, and the work by Brown et al<sup>13</sup> using HOE-140 would suggest that the observed effects are mediated principally through blockade of the B<sub>2</sub> receptor.

## Conclusions

B9340 is a potent, reversible, and selective competitive inhibitor of bradykinin-induced vasodilatation and t-PA release in humans. This compound is a potentially useful investigational tool in dissecting out the physiologic and pathophysiologic role of bradykinin in vivo in humans.

## Acknowledgments

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## **PUBLICATION 6**



# Inhibition of thrombin activatable fibrinolysis inhibitor augments fibrinolysis in human whole blood

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Exogenous thrombolytic therapy fails to achieve reperfusion of the infarct-related artery in approximately a third of patients with acute myocardial infarction [1]. Thrombin-activatable fibrinolysis inhibitor (TAFI) is a plasma procarboxypeptidase that may contribute to this resistance to thrombolysis. TAFI is converted to the active form, TAFIa, in the presence of activated coagulation factors, thrombin and thrombomodulin, and once activated, cleaves lysine groups from the C-terminal of partially degraded fibrin, thereby preventing the binding of plasminogen [2]. This greatly reduces the ability of tissue-type plasminogen activator (t-PA) to convert plasminogen to plasmin and inhibits the process of t-PA mediated fibrinolysis. Although elevated plasma concentrations of TAFIa have been reported during thrombolysis administration [3], it remains unclear whether TAFIa contributes to resistance to thrombolysis in man.

Potato carboxypeptidase inhibitor (PCI) is a specific inhibitor of TAFIa in blood that has been shown to accelerate t-PA mediated fibrinolysis both *in vitro* [2,4–6] and in animal models [7,8]. Previous *in vitro* studies, however, were based on the dynamics of preformed or *de novo* clot dissolution in the presence of specific blood products, or at best plasma. Thromboelastography is a global assay of fibrinolytic function that measures the viscoelastic properties of clotting blood over time and has been used to assess the fibrinolytic activity of exogenous thrombolytic agents in whole blood [9,10]. We investigated the effects of the specific TAFIa inhibitor, PCI, on t-PA mediated fibrinolysis using thromboelastography of human whole blood.

Studies were performed with the approval of the local research ethics committee and informed consent of each subject in accordance with the Declaration of Helsinki. Six healthy, male non-smokers aged 20–40 years were recruited. Using a 17-gauge needle, 9 mL venous blood was collected from an antecubital vein into plain, additive-free tubes (Sarstedt (Nümbrecht, Germany)) and immediately applied to a Thromboelastograph® Coagulation Analyser 5000C (Haemoscope Corporation, Skokie, IL, USA). Thromboelastography was

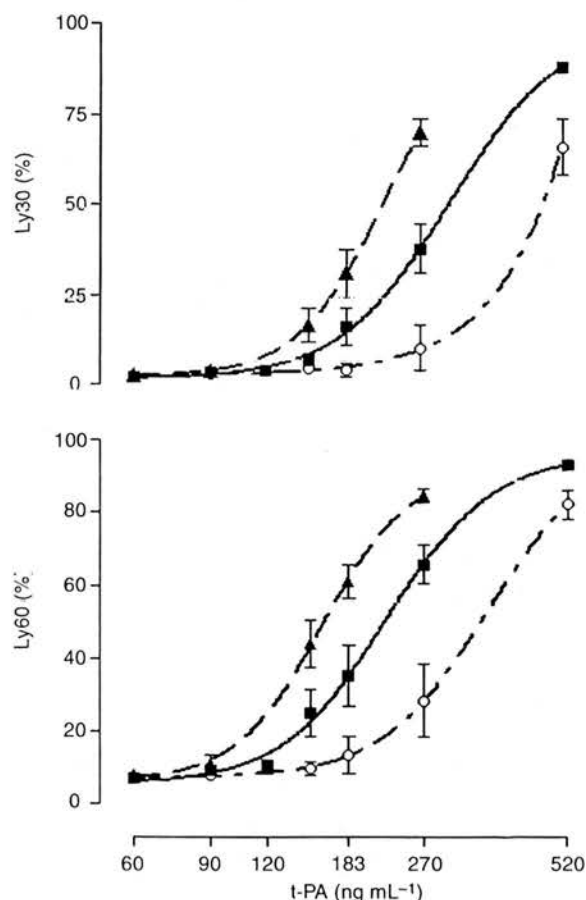
performed on 360 µL of samples consisting of 320 µL of whole blood, 10 µL of tissue factor (manufacturer's recommended concentration; Haemoscope Corporation) and 30 µL of normal saline containing the study drugs. Incremental doses of recombinant t-PA (final cup concentration: 0–560 ng mL<sup>-1</sup>; Boehringer Ingelheim, Ingelheim, Germany) were applied with saline placebo, PCI (27 µg mL<sup>-1</sup>; Calbiochem, Darmstadt, Germany) or tranexamic acid (420 ng mL<sup>-1</sup>; Pharmacia, Milton Keynes, UK) during tissue factor-initiated thromboelastography of human whole blood. Maximum amplitude (MA) and clot lysis at 30 (Ly30) and 60 (Ly60) minutes were determined using Thromboelastograph® software.

In the presence of saline placebo, t-PA caused a dose-dependent reduction in MA ( $P < 0.0001$ ; one-way ANOVA) and increases in Ly30 ( $P < 0.0001$ ; one-way ANOVA; Fig. 1) and Ly60 ( $P < 0.0001$ ; one-way ANOVA; Fig. 1). PCI augmented t-PA mediated lysis, causing a leftward shift in the t-PA dose response curve for both Ly30 and Ly60 ( $P < 0.001$  for both; PCI vs. placebo; Fig. 1). In contrast, tranexamic acid inhibited t-PA mediated lysis, shifting the t-PA dose response curves for Ly30 and Ly60 rightwards compared with placebo [ $P < 0.001$  for both; tranexamic acid (TA) vs. placebo; Fig. 1]. In the absence of t-PA, there were no significant differences in MA ( $57 \pm 2$  vs.  $58 \pm 2$  vs.  $57 \pm 1$  mm), Ly30 ( $2.5 \pm 0.9$  vs.  $2.0 \pm 0.7$  vs.  $2.9 \pm 0.8\%$ ) or Ly60 ( $7.0 \pm 0.9$  vs.  $6.1 \pm 1.2$  vs.  $7.4 \pm 1.3\%$ ) between saline placebo, PCI and tranexamic acid respectively.

We have demonstrated that inhibition of TAFIa augments t-PA mediated fibrinolysis in human whole blood. Thromboelastography has a number of potential advantages over traditional clot-based and turbidity-time course assays in that it allows incorporation of t-PA during, rather than after, thrombus formation [11] and is more likely to reflect physiological concentrations of TAFIa, given that TAFI activation occurs at sites of active clot formation where local thrombin concentrations are greatest [12]. It has been suggested that platelet derived TAFI may play a key role in the resistance of clot to thrombolysis [13]. Thromboelastography encompasses all of the constituents of blood involved in thrombus propagation including the cellular components absent from plasma-based assays. The demonstration that tranexamic acid, a competitive inhibitor of plasminogen activation, inhibits t-PA mediated fibrinolysis confirms the validity of thromboelastography as a method by which to assess fibrinolytic activity in human whole

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**Fig. 1.** Tissue factor initiated thromboelastography of human whole blood ( $n = 6$  for each data point): effect of incremental doses of t-PA [final cup concentration ( $\text{ng mL}^{-1}$ )] on Ly30 and Ly60 in the presence of saline placebo (solid squares), potato carboxypeptidase inhibitor (PCI,  $27 \mu\text{g mL}^{-1}$ ; solid triangles) or tranexamic acid (TA,  $420 \text{ ng mL}^{-1}$ ; open circles). Compared with placebo, PCI caused a significant leftward shift and tranexamic acid caused a significant rightwards shift in the Ly30 and Ly60 t-PA dose response curves ( $P < 0.001$  for all). Data are expressed as mean  $\pm$  SEM.

blood. We believe, therefore, that thromboelastography provides a robust and representative assessment of the clot dynamics anticipated at sites of thrombus formation in the human circulation.

To our knowledge, this is the first study to examine the functional effects of TAFIa inhibition in human whole blood. The concentrations of t-PA used in this study reflect plasma concentrations achieved during systemic thrombolysis for acute myocardial infarction (approximately  $100\text{--}200 \text{ ng mL}^{-1}$ ) [14]. Our findings are in keeping with previous data demonstrating PCI mediated augmentation of clot lysis in a plasma-based assay at similar concentrations of t-PA [6]. Moreover, consistent with our data, PCI had no effect on lysis at higher concentrations of t-PA ( $1000$  and  $5000 \text{ ng mL}^{-1}$ ), possibly as the authors suggest, because of loss of fibrin dependence [6].

Inhibition of TAFIa may also have a role in the secondary prevention of coronary heart disease. Low circulating endogenous fibrinolytic activity is associated with an increased risk of myocardial infarction in young men [15] and predicts which patients with unstable angina will progress to myocardial infarction [16]. Acute endothelial t-PA release is impaired in smokers [17] and in patients with coronary artery disease, and the degree of impairment correlates with atheromatous plaque load [18]. In patients with atheromatous vascular disease, TAFIa inhibition may augment endogenous fibrinolytic activity, swinging the balance in favor of clot dissolution rather than propagation, and interrupting the cycle of subclinical thrombus formation, organization and vessel remodeling that underlies chronic atheromatous plaque growth.

In summary, we have demonstrated that inhibition of TAFIa augments t-PA mediated fibrinolysis in human whole blood. Our findings provide functional evidence of TAFI activity in man and suggest that TAFIa inhibition may be both a potential adjuvant to thrombolytic therapy in patients with acute myocardial infarction, and a novel therapeutic strategy in the secondary prevention of thrombotic vascular events in patients with atherosclerosis.

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## Fast kinetic assay for the determination of procarboxypeptidase U (TAFI) in human plasma

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Carboxypeptidase U (CPU, TAFIa) is a recently discovered enzyme present in the circulation as its zymogen, procarboxypeptidase U (proCPU, TAFI). CPU was first described by Hendriks *et al.* [1,2] as an interference in the assay of carboxypeptidase N, a basic carboxypeptidase constitutively active in human plasma. After extensive research in the last 15 years, CPU is now regarded as an important link between coagulation and fibrinolysis [3–5]. Given that the proCPU concentration in plasma is far below its  $K_m$  for activation by the thrombin–thrombomodulin (T–Tm) complex the formation of CPU will be dependent both on the T–Tm concentration as well on the proCPU concentration [6,7]. A low or a high proCPU plasma concentration might therefore tip the balance between profibrinolytic and antifibrinolytic pathways and thereby cause a predisposition to bleeding or thrombosis. ProCPU levels in plasma can be measured using both immunological assays (ELISA) or activity-based assays. The intrinsic instability of CPU and the difference in stability

between isoforms are major drawbacks of activity-based assays and were solved in the past by using very high substrate concentrations (which stabilize the active enzyme), incubating for short periods of time or by incubating at room temperature [8,9]. Another possibility of solving this is making use of a kinetic method, which enables the determination of accurate initial velocities even at very low substrate concentrations. We recently published a kinetic assay for the efficient characterization of physiological and synthetic CPU substrates with a C-terminal arginine [8]. We now adapted this assay for use in a plasma environment. The reaction mechanism is shown in Fig. 1.

In order to determine plasma proCPU concentrations a reaction mixture is prepared consisting of 5.6 mmol L<sup>-1</sup> MgSO<sub>4</sub>, 22.2 mmol L<sup>-1</sup> KCl, 4.9 mmol L<sup>-1</sup> phosphoenolpyruvate, 1.1 mmol L<sup>-1</sup> NADH, 5.6 mmol L<sup>-1</sup> ATP 9000 U L<sup>-1</sup> of both pyruvate kinase and lactate dehydrogenase, 18 000 U L<sup>-1</sup> arginine kinase and 11 mmol L<sup>-1</sup> hippuryl-L-arginine substrate in HEPES buffer adjusted to pH 8.0. Prior to its addition to the reaction mixture, the pyruvate kinase/lactate dehydrogenase suspension is centrifuged for 5 min at 14 000 g, and the pellet is redissolved *ex tempore* in an equal amount of 100 mmol L<sup>-1</sup> HEPES buffer pH 8.0. Plasma is diluted 1/20 in 20 mmol L<sup>-1</sup> HEPES buffer pH 7.4 and activated in Eppendorf tubes (using final concentrations of T–Tm of 8 and 16 nmol L<sup>-1</sup>, respectively) and transferred to microtiter plates at room temperature. To 55 µL of activated plasma, 45 µL reaction mixture is added (37 °C). The microtiter plate is incubated for 3 min in a water bath at 37 °C to

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**PUBLICATION 7**

## Direct Vascular Effects of Protease-Activated Receptor Type 1 Agonism In Vivo in Humans

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**Background**—Protease-activated receptor type 1 (PAR-1) has been proposed as the principal thrombin receptor in humans, although its actions in vivo have not been defined. The aim of the present study was to determine the direct vascular actions of PAR-1 agonism in humans.

**Methods and Results**—Dorsal hand vein diameter was measured by the Aellig technique in 14 healthy volunteers during local intravenous SFLLRN (PAR-1 agonist; 0.05 to 15 nmol/min) and SLIGKV (PAR-2 agonist; 1.6 to 160 nmol/min) infusions. The venous effects of SFLLRN were further assessed in the presence or absence of norepinephrine or the glycoprotein IIb/IIIa antagonist tirofiban. Forearm blood flow was measured by venous occlusion plethysmography in 16 volunteers during infusion of SFLLRN (1 to 50 nmol/min), SLIGKV (160 to 800 nmol/min), and the endothelium-dependent vasodilator bradykinin (100 to 1000 pmol/min). Platelet-monocyte binding (a sensitive measure of platelet activation) and plasma tissue plasminogen activator (tPA), plasminogen-activator inhibitor 1, and von Willebrand factor concentrations were measured at intervals throughout the study. SFLLRN caused dose-dependent venoconstriction ( $P<0.001$ ) that was unaffected by norepinephrine or tirofiban co-infusion. In forearm resistance vessels, SFLLRN increased forearm blood flow ( $P<0.001$ ), tPA release ( $P<0.001$ ), and platelet-monocyte binding ( $P<0.0001$ ) without affecting plasma plasminogen-activator inhibitor 1 or von Willebrand factor concentrations. SLIGKV caused venous ( $P<0.001$ ) and arterial ( $P<0.01$ ) dilatation without tPA release.

**Conclusions**—We have demonstrated that PAR-1 agonism causes platelet activation, venous constriction, arterial dilatation, and tPA release in vivo in humans. These unique and contrasting effects provide important insights into the physiological and pathophysiological role of thrombin in the human venous and arterial circulations. (*Circulation*. 2006; 114:1625-1632.)

**Key Words:** platelets ■ blood flow ■ veins ■ arteries ■ receptor, PAR-1 ■ receptor, PAR-2

Thrombin is a powerful physiological stimulant in the cardiovascular system. Apart from its central enzymatic role in the coagulation cascade, it directly activates platelets, leukocytes, and vascular smooth muscle and endothelial cells.<sup>1</sup> Thrombin is therefore a vital link between thrombosis, cellular activation, and inflammation, key pathogenic factors in atherothrombotic disorders.

### Clinical Perspective p 1632

Given the apparent direct cellular effects of thrombin, cloning methods<sup>2,3</sup> were used to conduct an extensive search for its receptor. This led to the identification of G-protein-coupled protease-activated receptors (PARs) characterized by a unique mechanism of activation whereby proteolytic cleavage unmasks a short peptide sequence that remains tethered and activates the receptor.<sup>2,4</sup> Four different types of PARs have been identified. PAR-1, -3, and -4 are all activated by

thrombin. The type 1 receptor has been proposed as the principal thrombin receptor in humans.<sup>5,6</sup> In contrast, the PAR-2 receptor is activated by trypsin, by tryptase, and to a lesser extent by coagulation factors upstream of thrombin.<sup>7</sup> It appears to be of importance in inflammatory conditions that induce endothelial PAR-2 expression and vasodilatation.

PAR-1 receptor agonism has been extensively studied in vitro and is associated with platelet activation and aggregation,<sup>8</sup> vasodilatation,<sup>9</sup> and angiogenesis.<sup>10</sup> Although studies in small animals suggest that PAR-1 and PAR-2 agonism induces vasodilatation,<sup>11,12</sup> there is significant species heterogeneity, and rodent models are of limited relevance to humans.<sup>13</sup> Exploring the role of PAR-1 receptors in the human vasculature would deepen our understanding of the physiological role of thrombin and would be of interest in the development of new therapeutic strategies such as PAR-1 receptor antagonists<sup>14,15</sup> and direct thrombin inhibitors.<sup>16,17</sup>

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TABLE 1. Baseline Characteristics of Study Volunteers

Male, %	79 (41/52)
Age, y	24±0.5
Body mass index, kg/m <sup>2</sup>	23±0.4
Heart rate, bpm*	67±2
Blood pressure, mm Hg*	
Systolic	132±2
Diastolic	73±1
Hematocrit*	0.42±0.01
White cell count, ×10 <sup>9</sup> /L*	4.98±0.37
Platelets, ×10 <sup>9</sup> /L*	213±11

Values are mean±SEM.

\*Data from vascular studies only.

Therefore, the aims of the present study were to determine the direct in vivo vascular effects of PAR-1 agonism in the human vasculature. Specifically, we wished to assess the direct role of PAR-1 activation on platelet activation and aggregation, venous and arterial tone, and the release of endothelium-derived factors in vivo in humans.

## Methods

### Subjects

Fifty-two healthy male and female nonsmokers (age, 20 to 38 years) were recruited into the study (Table 1). Participants had not been taking any regular medications, over-the-counter medications, herbal supplements, or vitamins. They did not have clinically significant coexisting conditions, including hypertension, hyperlipidemia, diabetes mellitus, asthma, and coagulopathy, and had not suffered a recent infective or inflammatory condition. The study was approved by the local research ethics committee and conducted in accordance with the Declaration of Helsinki and with the written informed consent of all volunteers.

### Platelet Studies

#### Protocol 1: Platelet Aggregometry

Blood was sampled with a 19-gauge needle from the antecubital fossa into a 50-mL syringe, transferred into tubes containing 3.8% citrate, and centrifuged at 130g for 20 minutes at room temperature to obtain platelet-rich plasma. Blood was centrifuged at 1200g for 10 minutes to obtain platelet-poor plasma for reference samples. Platelet aggregation was measured at 37°C using standard optical platelet aggregometry.<sup>18,19</sup>

Preliminary in vitro platelet studies were carried out to construct concentration-response curves to define the concentration of the PAR-1 agonist SFLLRN-NH<sub>2</sub> that caused platelet activation and aggregation. SFLLRN-NH<sub>2</sub> (Auspep, Parkville Victoria, Australia) was compared with the thromboxane A<sub>2</sub> agonist U46619 (Sigma, Gillingham, UK) in both platelet-rich plasma and washed platelets.<sup>18</sup> To assess the specificity of the PAR-1-activating peptide, both SFLLRN-NH<sub>2</sub>- and U46619-induced platelet aggregation was assessed in the presence and absence of the PAR-1 antagonist RWJ-58259<sup>13</sup> ( $\alpha$ S-N-[(1S)-3-amino-1-[[[(phenylmethyl)-amino]carbonyl]propyl]-alpha-[[[1-(2,6-dichlorophenyl)methyl]-3-(1-pyrrolidinylmethyl)-1H-indazol-6-yl]amino]carbonyl]amino]-3,4-difluorobenzenepropanamide; American Peptide Co, Sunnyvale, Calif) in washed platelets. SFLLRN-NH<sub>2</sub>-induced platelet aggregation was also assessed in the presence of the direct thrombin inhibitor lepirudin (0.1 and 1.0 mg/mL; Schering, Burgess Hill, UK).

We chose the concentration of SFLLRN-NH<sub>2</sub> that caused nearly maximal platelet aggregation and assumed that the vascular and platelet PAR 1 receptor sensitivities were equivalent. However, before progressing to in vivo infusions, we defined the dose of tirofiban that would completely inhibit platelet aggregation using the

clinical-grade preparation of SFLLRN-NH<sub>2</sub>. Platelet-rich plasma was incubated for 5 minutes in the presence or absence of tirofiban (Merck, Sharp and Dohme, Hoddesdon, UK) at 50 ng/mL.<sup>20</sup> Platelet aggregation was measured after administration of 0.06 to 12  $\mu$ mol/L of the PAR-1-activating peptide SFLLRN-NH<sub>2</sub> (Clinalfa, Laufelfingen, Switzerland) by a 4-channel platelet aggregometry profiler (PAP-4D, Bio/Data Corp, Horsham, Pa) linked to PC interface software (Platelet Aggregation Profiler, version 2.3, Bio/Data Corp).

#### Protocol 2: Platelet-Monocyte Binding

Venous blood (5 mL) was collected through a 19-gauge needle and transferred into a tube containing the direct thrombin inhibitor D-phenylalanyl-L-propyl-L-arginine chloromethylketone. For in vitro studies, blood was collected into 2 D-phenylalanyl-L-propyl-L-arginine chloromethylketone tubes containing either tirofiban (50 ng/mL) or SFLLRN-NH<sub>2</sub> (0.06 to 6  $\mu$ mol/L). Five minutes after sampling, blood was incubated with appropriate monoclonal antibodies labeled with fluorochromes for 20 minutes, and platelet-monocyte aggregates were measured as described previously.<sup>21</sup>

### Vascular Studies

All studies were carried out in a quiet, temperature-controlled room (22°C to 24°C). Participants were semirecumbent (venous studies) or supine (arterial studies) and had abstained from alcohol for 24 hours and from food and caffeine-containing drinks for at least 4 hours before the study.

#### Venous Studies

A 23-gauge needle was sited in a dorsal hand vein, and the total infusion rate was kept constant at 0.25 mL/min in all studies. The hand was supported above the level of the heart and an upper arm cuff inflated to 40 mm Hg to obstruct venous return. The internal diameter of the dorsal hand vein was measured by the Aellig technique.<sup>22</sup> In brief, a magnetized lightweight rod rested on the summit of the infused vein  $\approx$ 1 cm downstream from the tip of the infusion needle. The rod passed through a core of a linear variable differential transformer, supported above the hand by a small tripod. Changes in vein diameter caused vertical displacement of the rod, leading to a linear change in the voltage generated by the linear variable differential transformer. This enabled calculation of absolute changes in vein size.

#### Protocol 3: Effect on Venous Tone

Because dorsal hand veins do not have resting tone, norepinephrine (1 to 128 ng/min) was used to induce and maintain a 50% to 70% reduction in vein diameter throughout the study to allow detection of either venodilation or venoconstriction. This was followed by co-infusion of the PAR-1-activating peptide SFLLRN-NH<sub>2</sub> (0.05 to 5 nmol/min) or the PAR-2-activating peptide SLIGKV-NH<sub>2</sub> (1.6 to 160 nmol/min; Clinalfa).

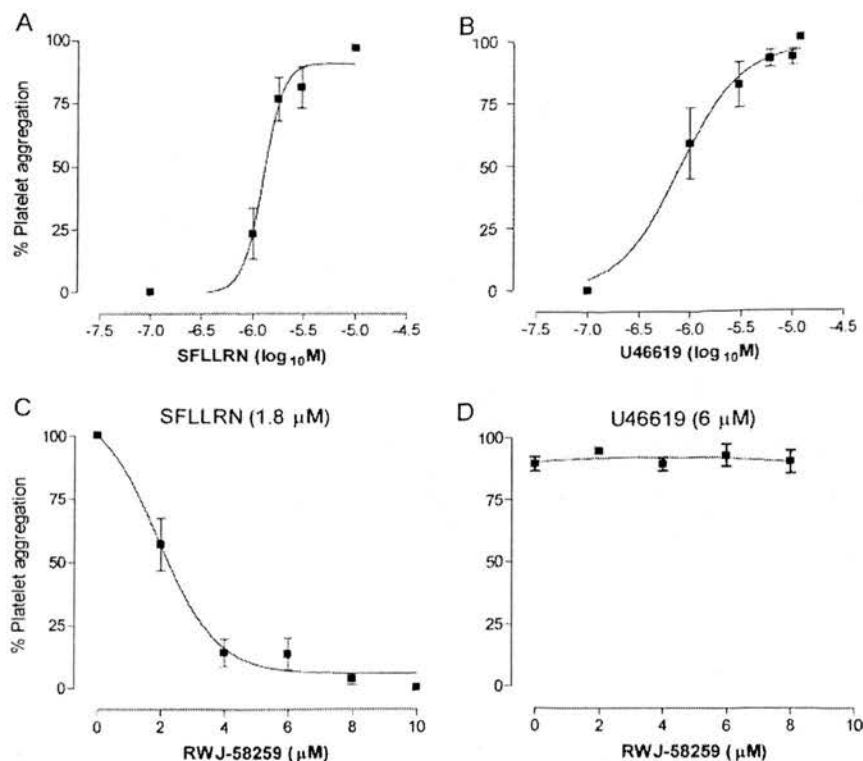
#### Protocol 4: Effect of Glycoprotein IIb/IIIa Receptor Antagonism

The effects of SFLLRN-NH<sub>2</sub> (0.05 to 15 nmol/min) in the presence and absence of the glycoprotein IIb/IIIa inhibitor tirofiban (250 ng/min) and norepinephrine (1 to 128 ng/min) were assessed to determine the importance of platelet aggregation on PAR 1 mediated alterations of venous tone. The doses of tirofiban and SFLLRN-NH<sub>2</sub> were chosen to achieve end-organ concentrations equivalent to those with efficacy in in vitro studies (protocol 1) and assumed a dorsal hand vein flow of 5 mL/min.

### Arterial Studies

All subjects underwent brachial artery cannulation with a 27-standard-wire-gauge steel needle under controlled conditions. Intrarterial infusion rate was kept constant at 1 mL/min throughout all studies. Forearm blood flow was measured in the infused and noninfused arms by venous occlusion plethysmography using mercury-in-Silastic strain gauges as described previously.<sup>23,24</sup> Supine heart rate and blood pressure were monitored at intervals throughout each study using a semiautomated noninvasive oscillometric sphygmomanometer. Tirofiban (1.25  $\mu$ g/min) was co-infused during PAR-1 activation to inhibit in vivo potential platelet aggre-





**Figure 1.** Induction of platelet aggregation by the PAR-1-activating peptide SFLLRN-NH<sub>2</sub> (A) and the thromboxane A<sub>2</sub> agonist U46619 (B;  $P < 0.001$  for both, 1-way ANOVA). The PAR-1 antagonist RWJ-58259 inhibited aggregation induced by SFLLRN-NH<sub>2</sub> (C;  $P < 0.001$ , 1-way ANOVA) but not by U46619 (D;  $P = \text{NS}$ ). Values are mean  $\pm$  SEM.

gation. The doses of tirofiban and SFLLRN-NH<sub>2</sub> were chosen to achieve end-organ concentrations equivalent to those with efficacy in in vitro studies (protocol 1) and assumed a brachial artery blood flow of 25 mL/min.

#### Blood Sampling

Venous cannulas (17 gauge) were inserted bilaterally into the antecubital fossae. Blood samples were drawn simultaneously from each arm during infusion of saline, tirofiban, and each dose of the PAR-activating peptides and bradykinin. They were collected into acidified buffered citrate (Stabilyte, Trinity Biotech Plc, Co Wicklow, Ireland; for tissue plasminogen activator [tPA] assays) and into citrate (BD Vacutainer, BD UK Ltd, Oxford, UK; for plasminogen activator inhibitor type 1 [PAI-1] and von Willebrand factor [vWF] assays). Samples were kept on ice before centrifugation at 2000g for 30 minutes at 4°C. Platelet-free plasma was decanted and stored at -80°C before assay. Plasma tPA antigen and activity (tPA Combi Actibind Elisa Kit, Technoclone, Vienna, Austria) and PAI-1 antigen and activity (ElitestPAI-1 Antigen and Zymutest PAI-1 Activity, Hyphen Biomed, Neuville-Sur-Oise, France) concentrations were determined by ELISAs. Full blood count was measured at baseline and at the end of the study. Blood was also collected from each arm to determine platelet-monocyte binding (see protocol 2) at baseline, at the highest dose of SFLLRN-NH<sub>2</sub>, during saline washout, and at the highest dose of bradykinin.

#### Protocol 5: PAR-1 Activation

After a 20-minute baseline saline infusion, tirofiban (1.25 μg/min) was infused throughout the study. Thirty minutes after the tirofiban infusion was started, the PAR-1-activating peptide SFLLRN-NH<sub>2</sub> was co-infused at 5, 15, and 50 nmol/min for 8 minutes at each dose separated by 6 minutes of saline washout infusions. This was followed by a 30-minute washout period before co-infusion of bradykinin (an endothelium-dependent vasodilator that releases tPA; Clinalfa) at 100, 300, and 1000 pmol/min<sup>25</sup> for 8 minutes at each dose.

#### Protocol 6: PAR-2 Activation

After a 20-minute baseline saline infusion, the PAR-2-activating peptide SLIGKV-NH<sub>2</sub> was infused at 160, 360, and 800 nmol/min

for 8 minutes at each dose separated by 6 minutes of saline washout infusions. This was followed by a 30-minute saline infusion before bradykinin was infused at 100, 300, and 1000 pmol/min<sup>25</sup> for 8 minutes at each dose.

#### Data and Statistical Analyses

Dorsal hand venous<sup>26</sup> and forearm plethysmographic<sup>23</sup> data were analyzed as described previously. Variables are reported as mean  $\pm$  SEM and analyzed using repeated-measures 1- or 2-way ANOVA with post-hoc Bonferroni corrections and 2-tailed Student *t* test as appropriate. Statistical analysis was performed with GraphPad Prism (GraphPad Software, Inc, San Diego, Calif). Statistical significance was taken at  $P < 0.05$ .

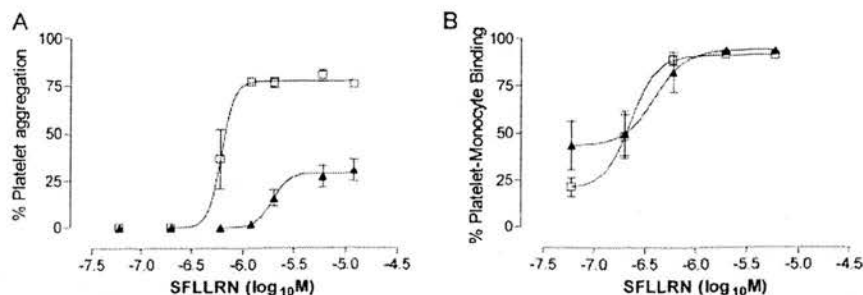
The authors had full access to the data and take responsibility for their integrity. All authors have read and agree to the manuscript as written.

## Results

### In Vitro Effects of PAR Agonism on Platelets

Both SFLLRN-NH<sub>2</sub> and U46619 caused dose-dependent platelet aggregation (median effective concentration [EC<sub>50</sub>] = 1.26 and 0.78 μmol/L, respectively; Figure 1A and 1B;  $n = 8$ ). The PAR-1 antagonist RWJ-58259 selectively and fully inhibited SFLLRN-NH<sub>2</sub>-induced (Figure 1C;  $n = 8$ ;  $P < 0.001$ , 1-way ANOVA) but not U46619-induced (Figure 1D;  $n = 8$ ;  $P = \text{NS}$ ) platelet aggregation. In contrast, the direct thrombin inhibitor lepirudin had no effect on SFLLRN-NH<sub>2</sub>-induced platelet aggregation ( $n = 6$ ;  $P = \text{NS}$ ; data not shown).

The clinical-grade SFLLRN-NH<sub>2</sub> also caused a dose-dependent increase in platelet aggregation (EC<sub>50</sub> = 0.66 μmol/L) that was inhibited by tirofiban (Figure 2A;  $n = 6$ ;  $P < 0.001$ , 2-way ANOVA). In contrast, PAR-1 activation caused a dose-dependent increase in whole-blood platelet-monocyte binding (EC<sub>50</sub> = 0.23 μmol/L) that was not affected by tirofiban (Figure 2B;  $n = 6$ ;  $P = \text{NS}$ , 2-way ANOVA). As anticipated, PAR-2



**Figure 2.** PAR-1-activating peptide (SFLLRN-NH<sub>2</sub>) induced platelet aggregation (A) and platelet-monocyte binding (B) in the presence (▲) or absence (□) of tirofiban (50 ng/mL). Values are mean  $\pm$  SEM. A,  $P < 0.0001$  (2-way ANOVA) vs tirofiban; B,  $P = NS$  (2-way ANOVA) vs tirofiban.

activation with SLIGKV-NH<sub>2</sub> induced neither platelet aggregation nor platelet-monocyte binding ( $n \geq 3$ ; data not shown).

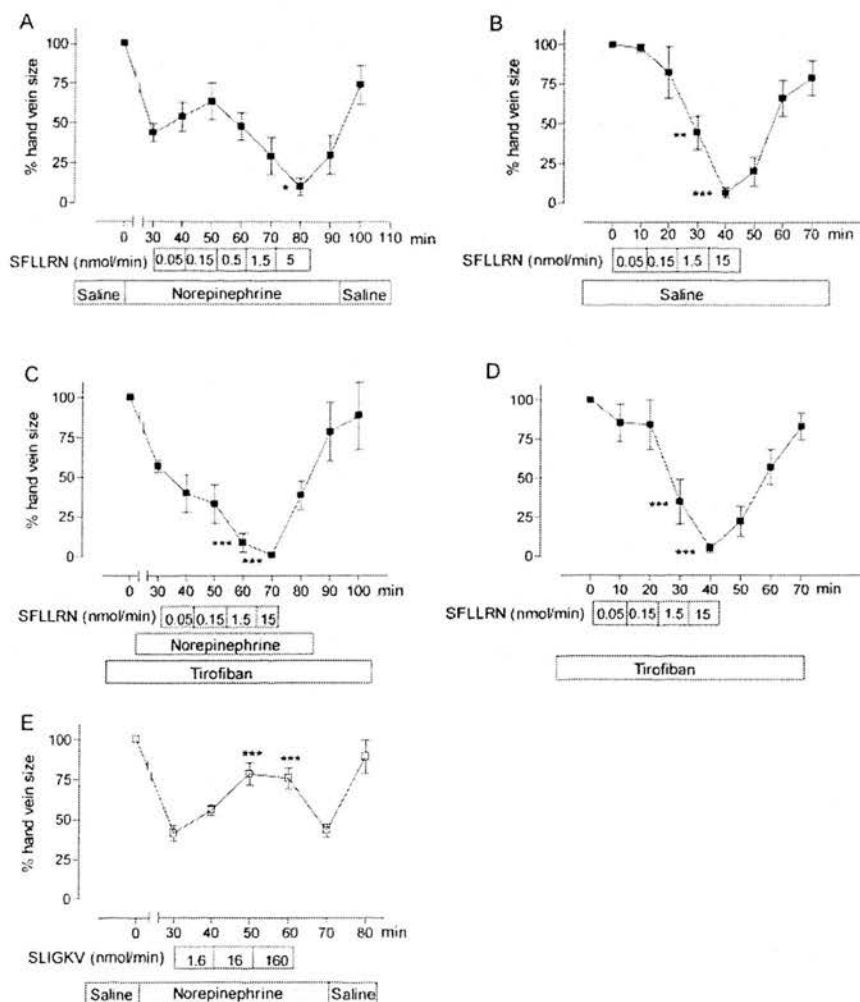
### In Vivo Effects of PAR Agonism on Dorsal Hand Veins

After venoconstriction (50% to 70%) was induced and maintained with norepinephrine, SFLLRN-NH<sub>2</sub> and SLIGKV-NH<sub>2</sub> caused dose-dependent venoconstriction ( $n = 8$ ;  $P < 0.001$ , 1-way ANOVA) and venodilatation ( $n = 6$ ;  $P < 0.001$ , 1-way ANOVA), respectively. In the absence of norepinephrine, SFLLRN-NH<sub>2</sub> caused a dose-dependent venoconstriction ( $P < 0.001$ , 1-way ANOVA) that was able to induce complete constriction of the venous segment. This was not mediated by platelet aggregation

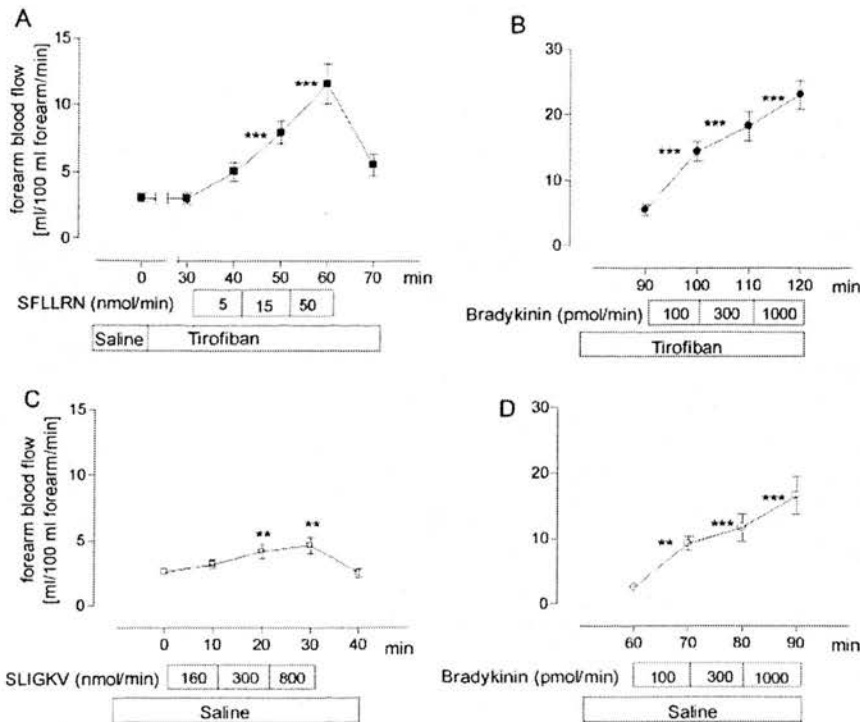
because co-infusion of tirofiban had no effect on SFLLRN-NH<sub>2</sub>-induced venoconstriction (Figure 3). SFLLRN-NH<sub>2</sub> was well tolerated by all subjects with no adverse effects. Vein patency was maintained at all times with no clinically apparent in situ thrombus formation.

### In Vivo Effects of PAR Agonism on Forearm Resistance Vessel Tone

There was no change in heart rate, blood pressure, or noninfused forearm blood flow throughout either study. Intra-arterial tirofiban had no effect on resting forearm blood flow ( $P = NS$ ). Both PAR-1 activation with SFLLRN-NH<sub>2</sub> and PAR-2 activation with SLIGKV-NH<sub>2</sub> caused dose-



**Figure 3.** The PAR-1-activating peptide SFLLRN-NH<sub>2</sub> causes concentration-dependent venoconstriction (■;  $n = 6$  to  $8$ ;  $P < 0.001$ , 1-way ANOVA) that is unaffected by norepinephrine (A, B) or tirofiban (C, D) infusion, whereas the PAR-2-activating peptide SLIGKV-NH<sub>2</sub> (E) induces venodilatation (□;  $n = 6$ ;  $P < 0.001$ , 1-way ANOVA). Values are mean  $\pm$  SEM. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .



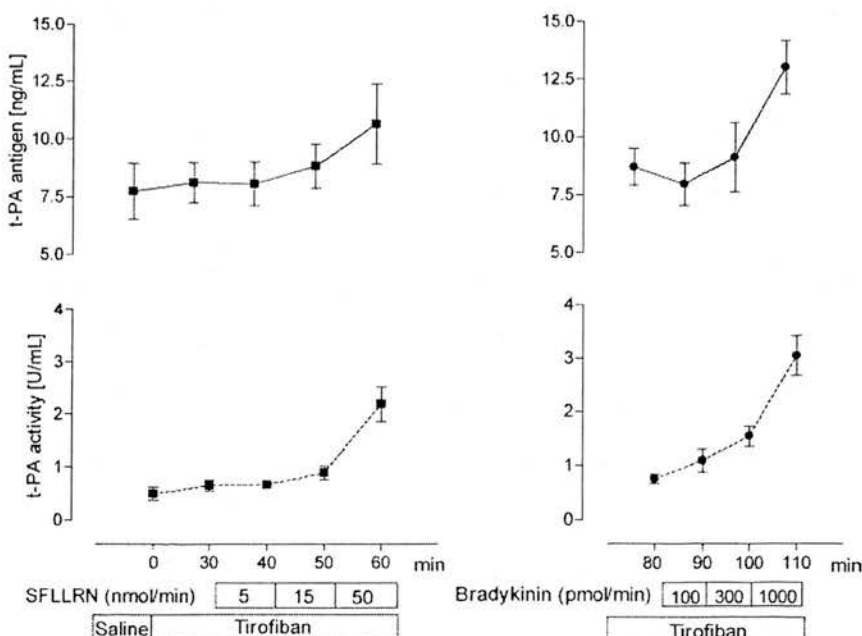
**Figure 4.** Forearm vasodilatation induced by the PAR-1-activating peptide SFLLRN-NH<sub>2</sub> (A; ■; n=8; *P*<0.001, 1-way ANOVA), the PAR-2-activating peptide SLIGKV-NH<sub>2</sub> (C; □; n=8; *P*<0.01, 1-way ANOVA), and bradykinin (B, ●; D, ○; n=8; *P*<0.001, 1-way ANOVA). Values are mean±SEM. \**P*<0.05, \*\**P*<0.01, \*\*\**P*<0.001.

dependent vasodilatation (Figure 4A and 4C; n=8; *P*<0.001 and *P*<0.01, respectively, 1-way ANOVA) with a rapid onset and offset of action. As anticipated, bradykinin increased forearm blood flow (Figure 4B; *P*<0.001, 1-way ANOVA) and was unaffected by tirofiban co-infusion (Figure 4D; *P*=NS versus no tirofiban).

### In Vivo Effects of PAR Agonism on Endothelium-Derived Factors

Plasma tPA antigen and activity concentrations increased in a dose-dependent manner during SFLLRN-NH<sub>2</sub> and bradykinin

but not SLIGKV-NH<sub>2</sub> infusion (Figure 5; *P*<0.001 for all). Plasma PAI-1 antigen and activity and vWF concentrations were unaffected by all infusions (*P*=NS for all; Table 2). Although there was an apparent rise in plasma PAI-1 antigen concentration at 50 nmol/min SFLLRN-NH<sub>2</sub>, it did not achieve statistical significance (*P*=0.08, paired *t* test versus baseline) and was not associated with increases in PAI-1 activity or vWF. There were no differences in peripheral blood hematocrit (0.416±0.006 versus 0.426±0.008) or platelet counts (213±16×10<sup>9</sup> versus 216±15×10<sup>9</sup>/L) at baseline and the end of the study. SFLLRN-NH<sub>2</sub> increased in



**Figure 5.** Plasma tPA antigen (solid lines) and activity (dashed lines) during infusion of the PAR-1-activating peptide SFLLRN-NH<sub>2</sub> (■) and bradykinin (●). Values are mean±SEM.

**TABLE 2. Plasma PAI-1 Antigen and Activity and vWF Antigen Concentrations in the Infused and Noninfused Arms During Brachial Artery Infusion of SFLLRN-NH<sub>2</sub> and Bradykinin**

	PAI-1 Antigen, ng/mL		PAI-1 Activity, U/mL		vWF Antigen, ng/mL	
	Infused Arm	Noninfused Arm	Infused Arm	Noninfused Arm	Infused Arm	Noninfused Arm
Baseline	27.3±5.5	27.7±5.6	0.58±0.14	0.51±0.13	0.72±0.08	0.72±0.07
Tirofiban	25.6±5.5	24.5±4.4	0.40±0.09	0.43±0.12	0.69±0.04	0.62±0.08
SFLLRN-NH <sub>2</sub> , nmol/min						
5	23.0±4.7	24.9±4.4	0.44±0.12	0.47±0.11	0.62±0.06	0.67±0.08
15	24.3±4.5	23.8±4.4	0.41±0.10	0.41±0.11	0.67±0.06	0.74±0.10
50	33.8±6.8	26.9±5.3	0.37±0.09	0.42±0.09	0.73±0.08	0.76±0.08
Washout	22.9±4.2	23.1±3.6	0.32±0.08	0.33±0.08	0.67±0.09	0.74±0.09
Bradykinin, pmol/min						
100	19.6±3.7	21.3±3.9	0.27±0.07	0.35±0.09	0.80±0.08	0.64±0.09
300	19.5±4.4	19.5±4.9	0.25±0.06	0.26±0.07	0.73±0.08	0.70±0.07
1000	19.5±3.2	19.1±2.9	0.16±0.03	0.25±0.05	0.76±0.07	0.67±0.07

n=8 for all. Values are mean±SEM.

vivo platelet-monocyte binding (from 16±5% to peak of 74±5%; Figure 6;  $P<0.0001$ , 1-way ANOVA) that declined toward baseline with time.

The PAR-2-activating peptide SLIGKV-NH<sub>2</sub> had no effect of plasma tPA concentrations ( $P=NS$  for all; data not shown).

## Discussion

In a series of clinical studies, we have, for the first time, described the direct vascular effects of PAR-1 agonism and established the induction of in vivo platelet activation, venoconstriction, vasodilatation, and tPA release, as well as confirming the vasodilatory effects of PAR-2 agonism. These unique and contrasting effects of PAR-1 activation provide important insights into the physiological and pathophysiological roles of thrombin in the venous and arterial circulations of humans.

### Platelet Effects of PAR-Activating Peptides

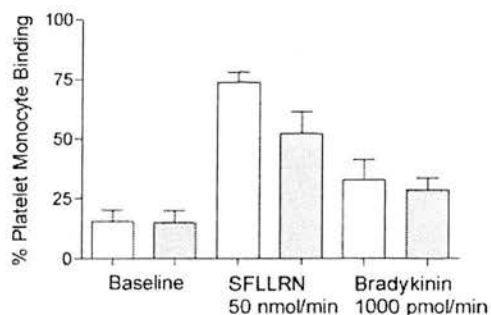
Inhibition of platelet activation has been of major benefit in the prevention and treatment of cardiovascular diseases. Thrombin is one of the most potent physiological stimulants of platelet activation that appears to be mediated largely through the PAR-1 receptor, although major contributions are also provided by PAR-4 and glycoprotein Ib receptors. To

date, strategies to reduce the action of thrombin have focused on direct inhibition of its enzymatic activity. Recently, PAR-1 antagonists have been developed as potential antiplatelet agents<sup>15</sup> that do not affect the coagulation cascade. It is therefore important to define the in vitro and in vivo effects of PAR-1 agonism in humans. As anticipated, we have demonstrated that PAR-1 but not PAR-2 agonism caused concentration-dependent platelet activation and aggregation. Moreover, in the presence of complete inhibition of platelet aggregation with the glycoprotein IIb/IIIa receptor antagonist tirofiban, PAR-1 agonism continued to induce platelet activation as measured by platelet-monocyte binding both in vitro and in vivo. This is consistent with our previous findings that although glycoprotein IIb/IIIa receptor antagonism inhibits platelet aggregation, it does not affect platelet activation or platelet-monocyte binding.<sup>27</sup>

### Vascular Effects of PAR-Activating Peptides

PAR-1 appears to be the principal receptor responsible for the vascular actions of thrombin and includes the regulation of vasomotion, platelet aggregation, inflammation, and angiogenesis. However, investigation of the in vivo vascular effects of thrombin presents challenges because of the direct activation of the coagulation cascade and platelets that may result in intravascular thrombosis. Here, we have chosen to use PAR-activating peptides that cause direct cellular stimulation without enzymatic activation of the coagulation cascade.

The direct vascular effects of PAR-1 receptor agonism have not been previously reported in humans. Our study protocol was vigilant to minimize any potential prothrombotic effects associated with PAR-1-induced platelet aggregation. We defined the in vitro concentrations of glycoprotein IIb/IIIa receptor antagonism that would permit the direct in vivo intravascular infusion of PAR-1-activating peptide without inducing platelet aggregation and potential thrombosis. This appears to have been successfully achieved, given the absence of significant adverse clinical side effects or thrombosis.



**Figure 6.** Platelet-monocyte binding during the infusion of the PAR-1 agonist SFLLRN-NH<sub>2</sub> in the infused (open bars) and non-infused (gray bars) arms. Values are mean±SEM.

### Venous Effects of PAR-Activating Peptides

Thrombin and the PAR-1 agonist SFLLRN-NH<sub>2</sub> have previously been shown to cause endothelium- and nitric oxide-dependent venous and arterial dilatation in canine and porcine ring segments.<sup>28,29</sup> Although confirming previous observations of PAR-2-mediated venodilatation,<sup>30</sup> we have rather unexpectedly described a dose-dependent venoconstriction with PAR-1 activation. This may be mediated by platelet activation, release of endothelium-derived vasoconstrictors, or a direct effect on venous smooth muscle cells. Neither norepinephrine nor tirofiban co-infusion appeared to affect this potent venoconstrictor effect. In combination with the apparent absence of in situ thrombosis, this suggests that PAR-1-induced venoconstriction was not mediated by platelet aggregation, although we cannot exclude an effect of platelet activation.

### Arterial Effects of PAR-Activating Peptides

In contrast to the venous effects, both PAR-1 and PAR-2 activation caused dose-dependent vasodilatation of forearm resistance vessels. We have not assessed the mechanism of this vasodilatation, but animal and clinical models suggest that this is likely to be endothelium and nitric oxide dependent.<sup>11,30</sup> However, data from animal studies have limited relevance in humans because of the wide species variability in PAR-1 receptor expression and function. The mechanism of PAR-1-induced vasodilatation needs to be addressed in future clinical studies.

PAR receptor expression varies between endothelial cell cultures originating from different human blood vessels,<sup>31</sup> which may partly explain the contrasting responses between PAR-1 and PAR-2 in the arterial and venous circulations. Our findings that PAR-2 activation causes venous and arterial dilatation are consistent with previous findings<sup>30</sup> and support the vascular role of PAR-2 receptors in inflammatory conditions.

### Effects of PAR-Activating Peptides on Endothelium-Derived Factors

Intra-arterial PAR-1 but not PAR-2 agonism caused an acute dose-dependent increase in local endothelial tPA release. In contrast to in vitro human endothelial culture studies,<sup>32</sup> this occurred in the absence of the release of other endothelium-derived factors such as PAI-1 and vWF. However, there was an apparent rise in plasma PAI-1 antigen ( $P=0.08$ ) with high-dose PAR-1 agonism, but given the absence of an effect on PAI-1 activity and vWF, this may reflect the associated platelet activation and release of platelet-derived PAI-1. PAI-1 is stored in platelet  $\alpha$ -granules, where its activity is  $\approx 5\%$  of plasma because of the absence of the stabilizing effect of vitronectin. Thus, PAR-1 agonism appears to have a selective profibrinolytic effect on the arterial endothelium.

### Physiological Significance of Vascular PAR-1 Activation

We have, for the first time, described the unexpected and contrasting vascular effects of PAR-1 agonism in vivo in humans. How do we interpret these effects? In an intact normal vessel, homeostatic mechanisms attempt to maintain vessel patency and minimize intravascular thrombus forma-

tion. Dorsal hand veins do not have resting tone, and the induction of venodilatation will not affect venous blood flow. Therefore, in the presence of developing venous thrombosis, venodilatation would not be beneficial, whereas venoconstriction will potentially limit thrombus propagation and embolization. In contrast, it would be anticipated that increasing blood flow and endogenous fibrinolysis would limit arterial thrombosis by ensuring rapid clearance and dissolution of a developing thrombus. We therefore propose that the vascular effects of PAR-1 agonism in vivo in humans can be understood in terms of limiting intravascular thrombosis and maintaining vessel patency. We speculate that these physiological effects may be disturbed in patients with cardiovascular disease or prothrombotic disorders.

### Conclusions

PAR-1 agonism causes platelet activation, venoconstriction, vasodilatation, and tPA release in vivo in humans. This has important implications in our understanding of the physiological vascular effects of thrombin and the pathogenesis of thromboembolic and atherothrombotic disorders.

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### Disclosures

None.

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### CLINICAL PERSPECTIVE

In addition to its well-known central enzymatic role within the coagulation cascade, thrombin is a powerful direct cellular activator of platelets, leukocytes, and vascular smooth muscle and endothelial cells. It is therefore a vital link between thrombosis and inflammation, key pathogenic factors in atherothrombotic disease. It has recently been discovered that thrombin causes direct cellular activation through a group of G-protein–coupled protease-activated receptors (PARs). Although the PAR-1 receptor is believed to mediate most of the receptor-mediated effects of thrombin, the cellular actions of PAR-1 activation have not been described in vivo in humans. In the present study, we establish for the first time the in vivo vascular effects of PAR-1 activation and demonstrate that it causes venous constriction, arterial dilatation, platelet activation, and release of endogenous tissue plasminogen activator. These intriguing and contrasting effects of PAR-1 agonism demonstrate the diverse nature of the vascular consequences of thrombin activation in humans. We suggest that, in the presence of a developing intravascular thrombosis, venoconstriction would limit thrombus propagation and embolization, whereas in the arterial circulation, increasing blood flow and endogenous fibrinolysis would limit arterial thrombosis by ensuring thrombus clearance and dissolution. We therefore propose that the vascular effects of PAR-1 agonism in vivo in humans can be understood in terms of limiting intravascular thrombosis and maintaining vessel patency. This has potential implications for applying novel PAR-1 receptor antagonists that are currently under clinical development as potential antiplatelet therapies.



## **PUBLICATION 8**

# Acute Effects of Glucocorticoids on Endothelial Fibrinolytic and Vasodilator Function in Humans

Anna R. Dover, PhD, Patrick W. F. Hadoke, PhD, Brian R. Walker, MD, and David E. Newby, PhD

**Abstract:** Acute coronary events occur most commonly in the morning, when circadian variations dictate that endogenous fibrinolytic activity is low and cortisol levels are high. We hypothesized that glucocorticoids would impair the acute fibrinolytic capacity of the endothelium because chronic glucocorticoid excess is associated with a prothrombotic state and endothelial vasomotor dysfunction. Twelve healthy subjects attended on 3 occasions and received oral metyrapone followed by intravenous saline or low-dose or high-dose hydrocortisone. Forearm blood flow and fibrinolytic indices were measured using venous occlusion plethysmography during intra-brachial bradykinin, acetylcholine, and sodium nitroprusside infusion.

Hydrocortisone infusion had no effect on systemic concentrations of plasminogen activator inhibitor type 1 (PAI-1) or tissue plasminogen activator (t-PA;  $P = 0.10$  and  $0.95$ , respectively). Bradykinin caused a dose-dependent increase in plasma t-PA concentrations ( $P < 0.0001$ ) that was unaffected by systemic hydrocortisone administration. Intrabrachial infusions of bradykinin, acetylcholine, and sodium nitroprusside all caused dose-dependent increases in forearm blood flow ( $P < 0.05$ ) that were unaltered by hydrocortisone infusions.

Short-term variations in plasma cortisol concentrations within the physiological range do not affect endothelial fibrinolytic or vasomotor function in healthy volunteers. These findings suggest that glucocorticoids do not exert acute effects on endothelial function *in vivo* in humans.

**Key Words:** glucocorticoids, endothelium, vasodilatation, fibrinolysis, human

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## INTRODUCTION

The incidence of acute coronary events exhibits circadian variation, peaking in the morning.<sup>1,2</sup> The mechanisms underlying this pattern are not understood but may include variations in endogenous fibrinolysis, either

systemically or locally within the blood vessel wall.<sup>3</sup> In particular, circadian fluctuations in the plasma concentrations, or activity, of circulating fibrinolytic factors, such as tissue plasminogen activator (t-PA), and their inhibitors [eg, plasminogen activator inhibitor type 1 (PAI-1)] result in attenuation of fibrinolysis in the morning.<sup>4</sup> Indeed, the efficacy of exogenous thrombolytic therapy also exhibits circadian variation, with treatment success (as judged by coronary arterial patency) achieved less commonly in the morning.<sup>5</sup>

The pattern of glucocorticoid secretion may contribute to the diurnal patterns of cardiovascular events and fibrinolytic function. Plasma cortisol concentrations are highest on waking and reach a nadir before sleep. Acute cardiac events and sudden cardiac deaths are more common in the aftermath of stressful environmental triggers (eg, earthquake<sup>6</sup>), when acute elevations of cortisol concentrations are predicted. Moreover, chronic glucocorticoid excess is associated with an increased incidence of cardiovascular events.<sup>7,8</sup> This may be due, at least partially, to direct effects of glucocorticoids on fibrinolysis, either systemically or locally at the vascular endothelium. Glucocorticoid excess, either endogenous or exogenous, is associated with elevations in PAI-1, von Willebrand factor, and factor VIII concentrations, favoring a hypofibrinolytic and hypercoagulable state.<sup>9–13</sup> Indeed, glucocorticoids enhance PAI-1 expression in cultured rat hepatocytes<sup>14</sup> and human adipose tissue,<sup>15</sup> which is significant because these organs may constitute major sources of plasma PAI-1.<sup>16,17</sup> Glucocorticoids potentiate vasoconstrictor responses to norepinephrine and angiotensin II,<sup>18,19</sup> and impair endothelium-dependent vasodilatation.<sup>20,21</sup> However, any acute influence of glucocorticoids on the capacity of the endothelium to release fibrinolytic factors, such as t-PA, has not been explored previously.

Here, we tested the hypothesis that acute elevations in plasma glucocorticoid concentrations (within the physiological range) adversely influence the systemic fibrinolytic balance and impair endothelial fibrinolytic and vasomotor function.

## MATERIALS AND METHODS

### Subjects

Twelve healthy non-smoking men were enrolled in the study, which was undertaken with the approval of the local research ethics committee, in accordance with the Declaration of Helsinki, and with the written informed consent of each subject. Subjects were excluded if they were taking vasoactive or antiinflammatory medication or if they had biochemical

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evidence of hyperglycemia or significant hepatic, renal, or thyroid dysfunction at an initial screening visit.

### Study Design

We aimed to compare basal physiological and moderately stressed levels of glucocorticoids with glucocorticoid depletion. Subjects attended on 3 occasions separated by a washout period of at least 1 week. On each occasion, subjects were permitted a light breakfast but refrained from all medications for 7 days, alcohol for 24 hours, and caffeine for at least 4 hours before the start of the study. In order to suppress endogenous adrenal glucocorticoid synthesis, subjects received 750 mg of 11 $\beta$ -hydroxylase inhibitor, metyrapone (Metopirone, Alliance Pharmaceuticals, UK) at midnight before the study day and again at 8:00 AM on the day of the study. Subjects attended for each study visit at 8 AM and rested recumbent in a quiet, temperature-controlled room. A 17-G cannula was inserted into the antecubital vein of each arm to allow blood sampling, and a dorsal foot vein was cannulated with a 23-G cannula for intravenous drug administration. Subjects received either (1) intravenous 0.9% saline (depletion of endogenous glucocorticoid), (2) low-dose (2.8 mg of priming bolus and 1.8 mg/hr infusion), or (3) high-dose (9.4 mg of priming bolus and 6.1 mg/hr infusion) intravenous hydrocortisone (Solu-Cortef, Pfizer UK) in a randomized, double-blind crossover design. During each phase, systemic t-PA and PAI-1 concentrations, local endothelial t-PA release and forearm vasodilator responses were measured between 3 and 5 hours of intravenous infusion as described below.

### Vasomotor Responses

Forearm venous occlusion plethysmography studies were commenced 3 hours after the initiation of intravenous drug administration. The brachial artery of the nondominant arm was cannulated with a 27-G steel needle (Cooper's Needle Works Ltd, Birmingham, UK) under 1% lignocaine (Xylocaine; Astra Pharmaceuticals Ltd, Kings Langley, UK) local anaesthesia. The cannula was attached to a 16-gauge epidural catheter (Portex Ltd, Hythe, UK) and patency was maintained by infusion of saline via an IVAC P1000 syringe pump (IVAC Ltd, Basingstoke, UK). The total rate of intraarterial infusion was maintained constant throughout all studies at 1 mL/min. After a 30-minute equilibration period (during which intraarterial saline was administered), subjects received intraarterial infusions of bradykinin at 100, 300, and 1000 pmol/min (bradykinin, an endothelium-dependent vasodilator which releases t-PA<sup>22,23</sup>; Merck Biosciences AG, L  ufelfingen, Switzerland), acetylcholine at 5, 10, and 20  $\mu$ g/min (acetylcholine, an endothelium-dependent vasodilator which does not release t-PA<sup>24,25</sup>; Novartis UK Ltd) and sodium nitroprusside at 2, 4, and 8  $\mu$ g/min (sodium nitroprusside, an endothelium-independent vasodilator<sup>24-27</sup>; David Bull Laboratories, UK). Each intraarterial drug was administered for 6 minutes (or 10 minutes in the case of bradykinin) at each dose, with drugs separated by 20-minute saline washout periods. Drugs were administered in random order, although the order was the same in all 3 phases for each subject. Forearm blood flow (FBF) was measured 3 minutes after the start of each drug dose and every 6 to 10 minutes throughout the washout periods.

Blood flow was measured in both forearms by venous occlusion plethysmography as previously described.<sup>27-29</sup> Mercury-in-silastic strain gauges applied to the widest part of the forearm were used to measure changes in forearm circumference.<sup>30</sup> During measurement periods, the hands were excluded from the circulation by rapid inflation of the wrist cuffs to a pressure of 200 mm Hg using E20 Rapid Cuff Inflators (D.E. Hokanson Inc, Washington, USA). Upper arm cuffs were inflated intermittently to 40 mm Hg pressure for 8 s in every 11 s to achieve venous occlusion and obtain plethysmographic recordings. Analogue voltage output from an EC-4 strain gauge plethysmograph (D.E. Hokanson) was processed by a PowerLab analogue-to-digital converter and Chart v4.1.2 for Windows software (AD Instruments Ltd, Castle Hill, Australia). Calibration was achieved using the internal standard of the plethysmograph. Blood pressure and pulse were monitored in the non-infused arm at intervals throughout each study using a semi-automated non-invasive oscillometric sphygmomanometer (Takeda UA 751, Takeda Medical Inc, Tokyo, Japan). Mean arterial pressure (MAP) was defined as the diastolic pressure plus a third of the pulse pressure.

### Endogenous Fibrinolytic Responses

Venous blood (8 mL) was withdrawn simultaneously from each arm at intervals throughout the study and collected into tubes containing potassium ethylene diamine tetraacetic acid (EDTA; Monovette, Sarstedt, N  mbrecht, Germany), lithium heparin (Monovette, Sarstedt, N  mbrecht, Germany), acidified buffered citrate (Biopool Stabilyte, Ume  , Sweden), and trisodium citrate (Monovette, Sarstedt, N  mbrecht, Germany) for estimation of haematocrit, cortisol, t-PA, and PAI-1, respectively. Citrate and acidified buffered citrate samples were centrifuged at 2000  $\times$  g for 30 min at 4  C, and lithium heparin samples at 1000  $\times$  g for 10 min at 20  C. Platelet-free plasma was decanted and stored at -80  C before assay.

Estimated net release of t-PA antigen was defined as the product of the infused forearm plasma flow (based on the hematocrit and the infused FBF) and the concentration difference between the infused arm ([t-PA]<sub>Inf</sub>) and noninfused arm ([t-PA]<sub>Non-inf</sub>) as previously described.<sup>27,31</sup>

### Laboratory Measurements

Plasma cortisol was determined using an enzyme-linked immunosorbent assay (DRG Instruments GmbH, Germany) with <2% crossreactivity for 11-deoxycortisol. Plasma PAI-1 and t-PA antigen concentrations and t-PA activities were determined as previously described<sup>32,33</sup> using enzyme-linked immunosorbent assays (Elitest PAI-1 antigen and Zymutest t-PA antigen; Hyphen-Biomed, France; Combi Actibind t-PA activity; Technoclone, UK). Haematocrit was determined using an automated Coulter counter (Beckman-Coulter ACT.8, High Wycombe, UK).

### Data Analysis and Statistics

Data were examined where appropriate by repeated measures analysis of variance (ANOVA) and post-hoc 2-tailed paired Student *t* test using GraphPad Prism (GraphPad Software, California, USA). Data are expressed as

mean  $\pm$  standard error of the mean. Differences were considered statistically significant if  $P < 0.05$ .

## RESULTS

The participants were men aged  $28 \pm 3$  years (age range, 21–48 years), with body mass index of  $25 \pm 1$  kg/m<sup>2</sup> and baseline random glucose concentrations of  $5.1 \pm 0.2$  mM. Oral, intravenous, and intra-arterial drugs were well tolerated with no adverse events.

### Plasma Cortisol Concentrations

An increase in plasma cortisol was evident after both low-dose and high-dose intravenous hydrocortisone compared with placebo ( $P < 0.0001$ ; Figure 1).

### Hemodynamic Effects

Baseline MAP, heart rate, and FBF were similar on all 3 visits (Table 1). There was no change in pulse during the studies, although mean arterial pressure rose during the study to a similar degree in all 3 phases (from  $88 \pm 4$  mm Hg to  $101 \pm 2$  mm Hg with placebo,  $89 \pm 3$  mm Hg to  $97 \pm 3$  mm Hg with low-dose hydrocortisone, and  $92 \pm 2$  mm Hg to  $101 \pm 2$  mm Hg with high-dose hydrocortisone;  $P < 0.001$  for all).

Bradykinin, acetylcholine, and sodium nitroprusside all caused dose-dependent increases in FBF in the infused arm without affecting blood flow in the noninfused arm (Figure 2). There were no differences among saline and the 2 hydrocortisone infusions in FBF during intraarterial infusion of bradykinin, acetylcholine, or sodium nitroprusside (Figure 2).

### Plasma Fibrinolytic Variables

Baseline plasma t-PA antigen concentrations and t-PA activity in the infused and noninfused arm were unchanged by systemic hydrocortisone infusion ( $P = 0.95$  for noninfused

TABLE 1. Baseline Hemodynamic Characteristics

	Placebo	Low-Dose Hydrocortisone	High-Dose Hydrocortisone	P Value*
Heart rate (/min)	67 $\pm$ 3	63 $\pm$ 3	68 $\pm$ 4	0.30
Mean Arterial Pressure (mm Hg)	88 $\pm$ 4	89 $\pm$ 3	92 $\pm$ 2	0.59
Forearm blood flow, infused (mL/100 mL/min)	2.0 $\pm$ 0.3	3.0 $\pm$ 0.5	2.4 $\pm$ 0.3	0.12
Forearm blood flow, non-infused (mL/100 mL/min)	1.8 $\pm$ 0.2	2.8 $\pm$ 0.4	2.3 $\pm$ 0.3	0.08

\*Repeated measures ANOVA comparing treatment phases.

arm; Figure 3). Bradykinin caused dose-dependent increases in infused arm total and active plasma t-PA concentrations that did not differ between treatment groups when comparing either plasma t-PA concentration (ng/mL,  $P = 0.74$ ; Figure 3) or estimated net t-PA release (ng/100 mL tissue/min;  $P = 0.88$ , Figure 3).

There was a trend towards an increase in infused arm plasma PAI-1 concentrations with increasing intravenous hydrocortisone dose that did not reach statistical significance ( $P = 0.10$  versus noninfused arm; Table 2). There was no significant change in plasma PAI-1 concentrations during intrabrachial bradykinin infusion in the infused ( $P = 0.45$ ) or noninfused ( $P = 0.71$ ) arm (Table 2).

## DISCUSSION

These data show that acute variations in plasma glucocorticoid concentrations alter neither the systemic fibrinolytic balance nor the capacity for the endothelium to release t-PA acutely in response to bradykinin. Furthermore, in contrast to prolonged administration of pharmacological glucocorticoids,<sup>20</sup> short-term manipulation of plasma glucocorticoid concentrations does not alter endothelial vasomotor function in vivo in humans.

### Vasomotor Function

Previous studies have shown that prolonged administration (5 days) of hydrocortisone results in hypertension and impairment of endothelium-dependent vasodilatation.<sup>20</sup> The effects of glucocorticoids on vascular responsiveness have been attributed to impairment of nitric oxide bioavailability,<sup>20,21,34</sup> although the exact mechanisms remain elusive. Glucocorticoids also potentiate vasoconstrictor responses to noradrenaline and angiotensin II.<sup>18,19</sup> The findings that cortisol can induce hypertension within 24 hours and that ACTH infusion produces an elevation in blood pressure within 2 to 8 hours<sup>35</sup> suggest that cortisol-mediated effects on vascular tone might be apparent in the acute setting. However, the present data suggest that, despite nearly 6-fold differences in plasma cortisol concentrations, no difference in endothelial vasomotor function was apparent over 3 to 5 hours. These findings are consistent with recently published data showing that systemic administration of pharmacological doses of hydrocortisone (200 mg infused over 3 hours) has no effect on plasma

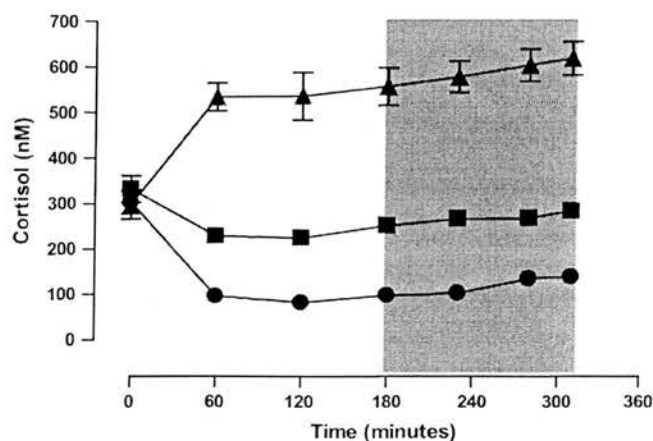
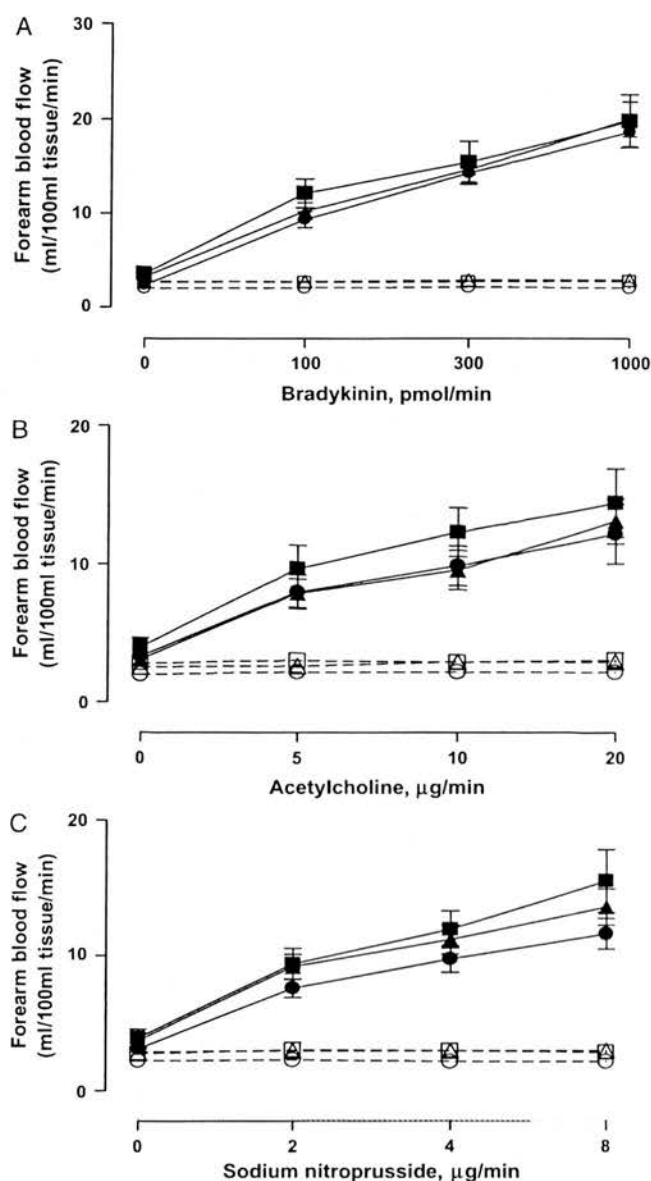


FIGURE 1. Plasma cortisol concentrations. Plasma cortisol concentration during the study in subjects ( $n = 12$ ) treated with intravenous saline (closed circles), low-dose hydrocortisone (closed squares) and high-dose hydrocortisone (closed triangles). Significant differences in plasma cortisol were observed among all 3 groups ( $P < 0.0001$ ). Shaded area represents forearm plethysmography study.





**FIGURE 2.** Forearm blood flow (FBF) responses to vasodilators. Infused (closed symbols) and noninfused (open symbols, dotted line) FBF in response to increasing doses of (A) bradykinin, (B) acetylcholine, and (C) sodium nitroprusside in subjects ( $n = 12$ ) treated with intravenous saline (circles), low-dose hydrocortisone (squares), and high-dose hydrocortisone (triangles).  $P < 0.001$  for FBF in infused arm,  $P = ns$  for comparisons among groups.

nitrate/nitrite activity or forearm vascular responsiveness to acetylcholine.<sup>36</sup> Taken together with evidence that direct intraarterial short-term cortisol infusion does not alter vascular resistance in the human forearm vascular bed,<sup>36,37</sup> these findings suggest that cortisol does not acutely alter basal vascular tone, nitric oxide generation, or vasodilator responses.

There are a number of possible explanations for the lack of effect of short-term hydrocortisone infusion on

endothelium-dependent vasodilatation. It may be that there are no direct effects of glucocorticoids on endothelial cell function in humans. This conclusion is supported by observations that in vitro incubation of human subcutaneous resistance arteries with cortisol ( $30 \mu\text{M}$  for 3 hours) had no effect on endothelium-dependent vascular reactivity (Hadoke PWF, unpublished observations). Alternatively, the acute effects of glucocorticoids on endothelial function may be mediated through de novo gene transcription, which takes more than 3 hours to become evident. Whilst glucocorticoid-mediated effects on the generation of reactive oxygen species by mononuclear cells<sup>38</sup> and on muscle sympathetic nerve activity are evident within 3 hours, effects on generation of reactive oxygen species and nitric oxide availability in human umbilical vein endothelial cells are manifest only after 6 hours.<sup>21</sup>

Metirapone was administered to all subjects in each treatment phase. Whilst it is possible that this drug had an effect on endothelial function that confounded the effects of glucocorticoids (for example, due to elevated levels of the precursor mineralocorticoid 11-deoxycortisol), this is unlikely because previous studies have shown no effect of this drug on either basal FBF in vivo or endothelial nitric oxide release in vitro.<sup>39</sup>

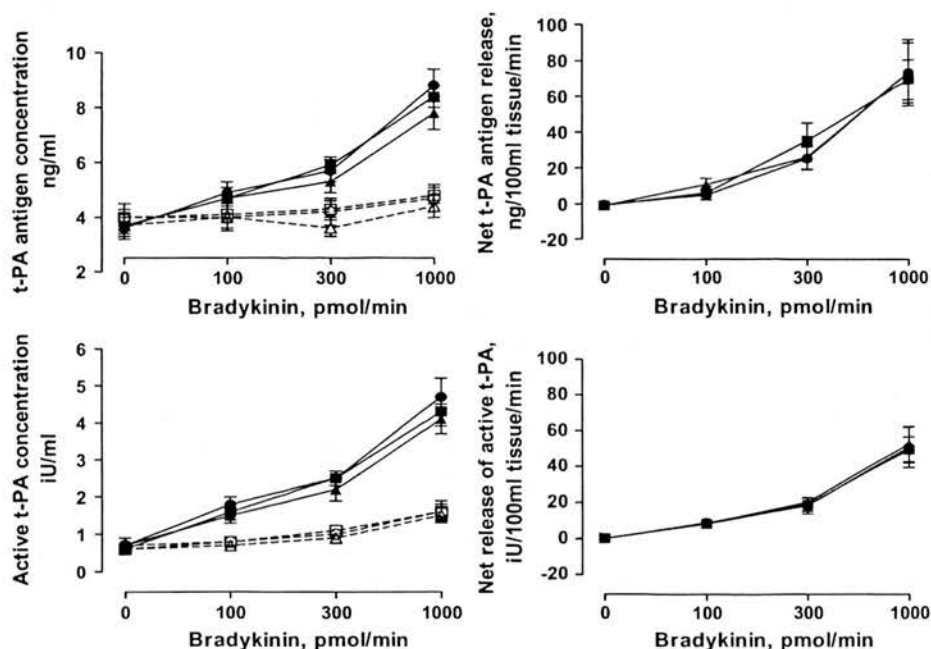
### Fibrinolytic Function

Although glucocorticoid excess has been associated with elevated plasma PAI-1 concentration and a hypofibrinolytic and hypercoagulable state,<sup>9–13</sup> there have been no studies to date investigating whether physiological variations in glucocorticoid concentrations influence the local fibrinolytic capacity of the endothelium. The mechanisms regulating acute t-PA release and plasma PAI-1 concentrations are likely to involve the nitric oxide pathway.<sup>28</sup> Augmentation of t-PA release is seen after acute inflammation,<sup>40</sup> and impairment of fibrinolytic activity is predictive of increased cardiovascular risk.<sup>41</sup> Considering the antiinflammatory properties of glucocorticoids, their ability to inhibit nitric oxide production by iNOS, and their association with cardiovascular risk, one might predict an adverse effect of glucocorticoids on the acute capacity of the endothelium to release t-PA. However, the present study has demonstrated that short-term systemic administration of hydrocortisone had no effect on basal or bradykinin-stimulated acute release of t-PA from the endothelium.

In addition to the lack of effect of hydrocortisone on acute t-PA release, we also found no change in plasma PAI-1 concentrations after short-term hydrocortisone administration. These results parallel those from a recent study that showed no change in plasma PAI-1 levels in healthy volunteers treated with dexamethasone ( $6 \text{ mg}$ ) daily for 5 days.<sup>42</sup> However, elevations in PAI-1 levels have been demonstrated in patients with endogenous glucocorticoid excess<sup>10</sup> and in those receiving long-term glucocorticoid therapy after heart<sup>13</sup> and renal transplantation.<sup>12</sup> These elevations in PAI-1 improve upon surgical correction of Cushing syndrome<sup>10</sup> or steroid withdrawal.<sup>9</sup> Furthermore, studies in vitro<sup>14,43–48</sup> and in animal models in vivo<sup>49,50</sup> have also demonstrated an augmentation of PAI-1 release after glucocorticoid treatment. The lack of effect



**FIGURE 3.** t-PA concentrations during intrabrachial bradykinin infusion. Plasma concentrations (ng/mL; left panels) and estimated net release (ng/100mL tissue/min; right panels) of total (ng) and active (iU) t-PA antigen in infused (closed symbols) and noninfused (open symbols, dotted line) arms during intrabrachial bradykinin infusion in subjects treated with intravenous saline (circles), low-dose hydrocortisone (squares), and high-dose hydrocortisone (triangles).  $P < 0.001$  for t-PA dose response in infused arm and net release.  $P = ns$  for between group comparisons.



of short-term hydrocortisone on PAI-1 concentrations in our study suggests that, in contrast to studies of chronic steroid excess and studies in vitro, PAI-1 is not released in response to hydrocortisone over the time course and at the physiological doses used in the present study.

## CONCLUSIONS

We conclude that short-term variations in plasma glucocorticoid levels within the physiological range do not alter the systemic fibrinolytic balance or endothelial fibrinolytic and vasomotor function. If there are direct effects of glucocorticoids on endothelial cell function in vivo, it may be that they require a longer duration to become manifest. It therefore appears unlikely that circadian variations in cortisol are responsible for variations in fibrinolytic function, although it remains possible that either of these pathways contribute to the circadian variation in cardiovascular disease events.

**TABLE 2.** Plasma PAI-1 Concentrations Before and During Bradykinin Administration After Intravenous Placebo or Hydrocortisone

Arm	0 pmol/min Bradykinin	1000 pmol/min Bradykinin
Placebo		
Infused (ng/mL)	18.8 ± 1.7	18.5 ± 1.6
Noninfused (ng/mL)	19.3 ± 1.0	19.9 ± 2.7
Low-dose hydrocortisone		
Infused (ng/mL)	22.2 ± 2.9	21.7 ± 1.9
Noninfused (ng/mL)	20.9 ± 2.6	24.3 ± 2.0
High-dose hydrocortisone		
Infused (ng/mL)	26.4 ± 3.5	24.0 ± 3.0
Non-infused (ng/mL)	24.0 ± 4.1	24.7 ± 3.3

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## **PUBLICATION 9**

## Endothelial Function

# Acute Systemic Inflammation Enhances Endothelium-Dependent Tissue Plasminogen Activator Release in Men

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<b>OBJECTIVES</b>	The purpose of this study was to investigate in vivo the effects of acute systemic inflammation on the endogenous fibrinolytic capacity in men.
<b>BACKGROUND</b>	Systemic inflammation and endogenous fibrinolysis play a major role in the pathogenesis of coronary artery disease. Although previous studies have shown impaired endothelium-dependent vasomotor function, the effects of inflammation on the endothelial release of the fibrinolytic factor tissue plasminogen activator (t-PA) are unknown.
<b>METHODS</b>	In a double-blind randomized placebo-controlled crossover trial, we administered a mild inflammatory stimulus, <i>Salmonella typhi</i> vaccine, or saline placebo to eight healthy men on two separate occasions. Six hours after vaccination, blood flow and plasma fibrinolytic variables were measured in both arms during intrabrachial infusions of bradykinin (40 to 1,000 pmol/min), acetylcholine (5 to 20 $\mu$ g/min), and sodium nitroprusside (2 to 8 $\mu$ g/min).
<b>RESULTS</b>	Compared with placebo, the <i>S. typhi</i> vaccination caused a rise in white cell count ( $11.1 \pm 0.5 \times 10^9/l$ vs. $7.9 \pm 0.8 \times 10^9/l$ ; $p = 0.004$ ) and plasma interleukin-6 concentrations ( $6.9 \pm 1.4$ pg/ml vs. $1.6 \pm 0.4$ pg/ml; $p = 0.01$ ) in addition to a significant augmentation of t-PA antigen ( $45 \pm 9$ ng/100 ml/min at peak dose vs. $24 \pm 8$ ng/100 ml/min at peak dose; $p = 0.016$ , analysis of variance) and activity ( $104 \pm 15$ IU/100 ml/min vs. $54 \pm 12$ IU/100 ml/min; $p = 0.006$ , analysis of variance) release during bradykinin infusion. Forearm blood flow increased in a dose-dependent manner after bradykinin, acetylcholine and sodium nitroprusside infusions ( $p < 0.001$ ), but this was unaffected by vaccination.
<b>CONCLUSIONS</b>	Our results showed that acute systemic inflammation augmented local forearm t-PA release in men, which suggests that acute inflammation may invoke a protective response by enhancing the acute endogenous fibrinolytic capacity in healthy men. Further studies are needed to clarify whether this response is impaired in patients with cardiovascular disease. (J Am Coll Cardiol 2003;41:333-9) © 2003 by the American College of Cardiology Foundation

Atherosclerosis is widely recognized to be an inflammatory disease process involving dysfunction of the vascular endothelium (1). This dysfunction leads to increased expression of leukocyte adhesion molecules, reduced anticoagulant activity and the release of growth factors, inflammatory mediators, and cytokines. Continued inflammation leads to leukocyte and monocyte recruitment, arterial damage, and atherogenesis. Additional cycles of damage cause plaque expansion and disruption that may lead to angina, crescendo angina, and acute coronary syndromes.

Recent epidemiological and observational studies have suggested a link between systemic inflammation and coronary artery disease. Infections by organisms, such as *Chlamydia pneumoniae* and herpes simplex virus type 1, appear to

be associated with an increased risk of cardiovascular mortality (2), and approximately 4% of bacteremic patients will develop acute myocardial infarction within a month of infection (3). Increased cardiovascular mortality also is seen after respiratory tract infections (4), severe illnesses requiring intensive care (5), and surgery (6). Markers of systemic inflammation, such as C-reactive protein (CRP), serum amyloid A, interleukin-6 (IL-6), and tumor necrosis factor- $\alpha$ , are elevated in patients with cardiovascular disease and are associated with an adverse prognosis and recurrent coronary events (7-10). Moreover, in previously healthy individuals, elevated plasma CRP and IL-6 concentrations predict the development of cardiovascular disease (11-13). Indeed, reflecting its anti-inflammatory action, the preventative benefits of aspirin in reducing cardiovascular risk are proportional to the plasma CRP concentration (11). These data collectively suggest two patterns of association: a link between chronic inflammation and the slow process of atherogenesis and an association between acute systemic inflammation and a transiently increased risk of an acute cardiovascular event.

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#### Abbreviations and Acronyms

ANOVA	= analysis of variance
CRP	= C-reactive protein
FBF	= forearm blood flow
IL-6	= interleukin-6
PAI-1	= plasminogen activator inhibitor type 1
t-PA	= tissue-type plasminogen activator

The vascular endothelium plays a vital role in the control of blood flow, hemostasis, fibrinolysis, and inflammation (14), and impaired endothelial function is implicated in the pathogenesis of coronary artery disease. Tissue plasminogen activator (t-PA) is a fibrinolytic factor released from the endothelium through the translocation of a dynamic intracellular storage pool and regulates the degradation of intravascular fibrin (15). If endogenous fibrinolysis is to be effective, then the rapid mobilization of t-PA from the endothelium is essential because thrombus dissolution is much more effective if t-PA is incorporated during, rather than after, thrombus formation (16,17). However, in the presence of pro-inflammatory states or an imbalance in endogenous fibrinolysis, such microthrombi may propagate, ultimately leading to arterial occlusion and tissue infarction (18).

We have previously described an *in vivo* model to assess the acute release of t-PA in men (19) and demonstrated an association between t-PA release and endothelial dysfunction (20,21). Hingorani et al. (22) also have recently shown that acute inflammation causes dysfunction of endothelium-dependent vasodilation in humans. However, there have been no studies to assess directly the acute local fibrinolytic capacity after acute inflammation. Therefore, the aim of this study was to test the hypothesis that the acute fibrinolytic capacity is altered by a mild systemic inflammatory response generated by typhoid vaccination.

## METHODS

**Subjects.** Eight healthy nonsmoking men between 20 and 27 years of age participated in the study, which was undertaken with the approval of the local research ethics committee and in accordance with the Declaration of Helsinki. The written informed consent of each subject was obtained before entry into the study.

All subjects were normotensive without a history of diabetes mellitus and vascular or coronary artery disease. None of the subjects had undergone typhoid vaccination in the previous year or received vasoactive or nonsteroidal anti-inflammatory drugs in the week before the study. All subjects abstained from alcohol for 24 h and from food and caffeine-containing drinks for at least 4 h before each forearm study. All studies were performed in a quiet, temperature-controlled room maintained at 22 to 25°C.

**Drugs.** An inflammatory response was generated with a typhoid vaccination using *Salmonella typhi* capsular polysac-

charide vaccine 0.025 mg (Typhim Vi, Aventis Pasteur MSD, Berkshire, United Kingdom). Pharmaceutical-grade bradykinin (Clinalfa, Läufelfingen, Switzerland), acetylcholine (Clinalfa), and sodium nitroprusside (David Bull Laboratories, Faulding, United Kingdom) were administered after dissolution in saline (0.9%; Baxter Healthcare Ltd., Berkshire, United Kingdom). All solutions were freshly prepared on the day of study.

**Hemodynamic measurements.** Blood flow was measured in both forearms by venous occlusion plethysmography using mercury-in-silastic strain gauges as previously described (19-21). Blood pressure was monitored in the noninfused arm at intervals throughout each study with a semiautomated noninvasive oscillometric sphygmomanometer (Takeda UA 751, Takeda Medical Inc., Tokyo, Japan).

**Blood sampling and assays.** Blood was withdrawn simultaneously from each arm and collected into acidified buffered citrate (Biopool Stabilyte, Umeå, Sweden) for t-PA assays and trisodium citrate, ethylene diamine tetraacetic acid, and serum bead tubes (Monovette, Sarstedt, Nümbrecht, Germany) for plasminogen activator inhibitor type 1 (PAI-1), IL-6, and CRP assays, respectively, and kept on ice before being centrifuged at 2,000g for 30 min at 4°C. Platelet-free plasma was decanted and stored at -80°C before assay. Plasma t-PA, PAI-1, IL-6, and CRP concentrations were determined using specific enzyme linked immunosorbent assays (Coaliza t-PA and Coaliza PAI-1, Chromogenix AB, Mölndal, Sweden; Quantikine human IL-6 immunoassays, R&D Systems, United Kingdom; C-reactive protein enzyme-linked immunosorbent assay, Eurogenetics, Belgium, respectively) and plasma t-PA activity using a photometric method (Coatest t-PA, Chromogenix AB, Mölndal, Sweden). Hematocrit and white cell count were determined using an automated Coulter counter (Beckman-Coulter ACt.8 Coulter Counter, High Wycombe, United Kingdom).

**Study design.** The *S. typhi* vaccine or saline placebo were injected into the deltoid muscle of each subject's dominant arm at 8:30 AM in a randomized, balanced block, double-blind crossover manner at least two weeks apart. Previous reports have indicated that vaccination-induced endothelial dysfunction is transient and resolves within 32 h (22).

Six hours after vaccination and after a 4-h fast, strain gauges and cuffs were applied. The brachial artery of each subject's nondominant arm was cannulated with a 27-standard wire gauge steel needle (Cooper's Needle Works Ltd., Birmingham, United Kingdom) under 1% lidocaine (Xylocaine; Astra Pharmaceuticals, Wayne Pennsylvania) local anesthesia and attached to a 16-gauge epidural catheter (Portex Ltd., Hythe, United Kingdom). Patency was maintained by infusion of saline via a MS2000 syringe infusion pump (Graesby Medical, Watford, United Kingdom). Venous 17-gauge cannulae were inserted into large subcutaneous veins of the antecubital fossae of both arms. Forearm blood flow (FBF) was measured every 6 to 10 min. After 30 min of equilibration with saline infusion, intra-arterial



bradykinin was administered at 40, 200, and 1,000 pmol/min for 10 min at each dose, acetylcholine at 5, 10, and 20  $\mu\text{g}/\text{min}$ , and sodium nitroprusside at 2, 4, and 8  $\mu\text{g}/\text{min}$  for 6 min at each dose. The drugs were separated by 20 min of saline infusion and administered in a randomized order that was kept constant for each individual. Venous samples were taken at baseline and during infusion of each bradykinin dose but not during sodium nitroprusside or acetylcholine infusion because they do not affect plasma t-PA or PAI-1 concentrations in this forearm model (19,23). White cell count, hematocrit, IL-6, and CRP were determined 6 h after vaccination and hematocrit was repeated at the end of the forearm study.

**Data analysis and statistics.** Plethysmographic data were extracted from the chart data files, and FBF was calculated for individual venous occlusion cuff inflations by use of a template spreadsheet (Microsoft Excel 97). Recordings for the first 60 s after wrist cuff inflation were not used because of the variability in blood flow this initial inflation causes. The last five linear plethysmographic recordings in each 3-min measurement period were calculated and averaged for each arm. The estimated net release of t-PA antigen and activity was defined previously (19) as the product of the infused forearm plasma flow (based on the mean hematocrit and the infused FBF) and the concentration difference between the infused ( $[\text{t-PA}]_{\text{inf}}$ ) and noninfused ( $[\text{t-PA}]_{\text{noninf}}$ ) arms and is shown as follows:

$$\text{Estimated net t-PA release} = \text{FBF} \times (1 - \text{Hct}) \times ([\text{t-PA}]_{\text{inf}} - [\text{t-PA}]_{\text{noninf}})$$

The area under the curve (AUC) was calculated for estimated net t-PA antigen and activity release during bradykinin infusion.

Data were examined, where appropriate, by analysis of variance (ANOVA) with repeated measures and two-tailed paired Student *t* test using Excel 97 (Microsoft). All results are expressed as mean  $\pm$  SEM. Statistical significance was assigned at the 5% level.

## RESULTS

**Inflammatory response.** All subjects remained healthy throughout the study and reported no localized discomfort or systemic side effects after vaccination. Compared with placebo injection, there was a marked elevation in white cell count ( $11.1 \pm 0.5 \times 10^9/\text{l}$  vs.  $7.9 \pm 0.8 \times 10^9/\text{l}$ ;  $p = 0.004$ , *t* test) and plasma IL-6 concentrations ( $6.9 \pm 1.4 \text{ pg/ml}$  vs.  $1.6 \pm 0.4 \text{ pg/ml}$ ;  $p = 0.01$ , *t* test) 6 h after typhoid vaccination, although serum CRP concentrations ( $1.8 \pm 1.2 \text{ } \mu\text{g/ml}$  vs.  $1.0 \pm 0.6 \text{ } \mu\text{g/ml}$ ;  $p = \text{NS}$ , *t* test) and temperature ( $p = \text{NS}$ , *t*-test) (Table 1) were unchanged.

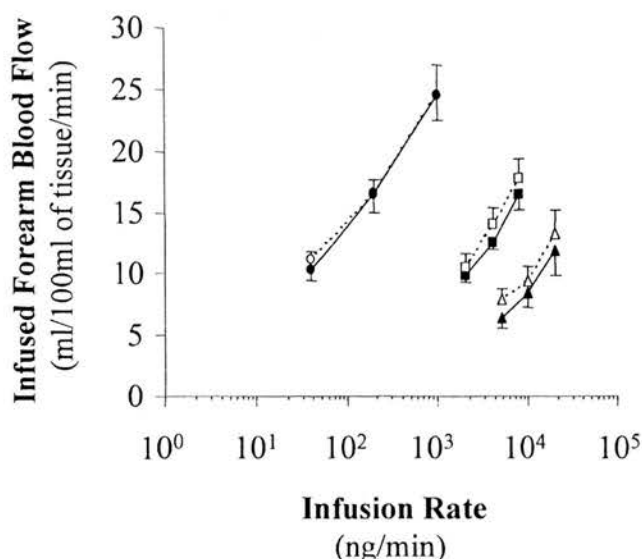
**Assessment of endothelium-dependent vasomotion.** There were no significant effects of vaccination on heart rate, blood pressure, or baseline FBF. There were no significant changes in heart rate, blood pressure, or noninfused FBF during drug infusion on either study day.

**Table 1.** Baseline Characteristics

	Vaccination	Placebo
Temperature ( $^{\circ}\text{C}$ )	$37.0 \pm 0.1$	$36.9 \pm 0.1$
Systolic blood pressure (mm Hg)	$131 \pm 4$	$129 \pm 5$
Diastolic blood pressure (mm Hg)	$62 \pm 3$	$66 \pm 3$
Heart rate (beats/min)	$57 \pm 1$	$57 \pm 2$
Hematocrit	$0.41 \pm 0.01$	$0.41 \pm 0.01$
Infused forearm blood flow (ml/100 ml tissue/min)	$3.0 \pm 0.6$	$2.8 \pm 0.6$

Forearm blood flow increased in a dose-dependent manner during bradykinin, acetylcholine, and sodium nitroprusside infusions ( $p < 0.001$ , ANOVA), but there was no change in the blood flow response after vaccination ( $p = \text{NS}$ , ANOVA) (Fig. 1).

**Assessment of fibrinolytic activity.** After vaccination, there were no changes in baseline plasma t-PA and PAI-1 antigen concentrations nor plasma t-PA activity concentration. Compared with the noninfused arm, bradykinin caused dose-dependent increases in plasma t-PA antigen and activity ( $p < 0.001$  for both, ANOVA) concentrations in the infused arm that were significantly higher after vaccination ( $p < 0.03$ , ANOVA) (Table 2). The *S. typhi* vaccination caused a significant augmentation in the net release of t-PA antigen ( $45 \pm 9$  vs.  $24 \pm 8 \text{ ng/100 ml/min}$  at peak dose;  $p = 0.016$ , ANOVA) and activity ( $104 \pm 15$  vs.  $54 \pm 12 \text{ IU/100 ml/min}$ ;  $p = 0.006$ , ANOVA) during bradykinin infusion, and a two- to threefold increase in the AUC for net t-PA antigen ( $37 \pm 12 \text{ ng/100 ml/min}$  vs.  $13 \pm 9 \text{ ng/100 ml/min}$ ;  $p = 0.14$ , *t* test) and activity ( $70 \pm 12 \text{ IU/100 ml/min}$  vs.  $36 \pm 8 \text{ IU/100 ml/min}$ ;  $p = 0.037$ , *t* test) release compared with placebo (Fig. 2). Plasma PAI-1



**Figure 1.** Infused forearm blood flow during bradykinin (circles), acetylcholine (triangles), and sodium nitroprusside (squares) infusions in subjects who were administered typhoid vaccination (closed symbols) and saline placebo (open symbols). Analysis of variance,  $p < 0.001$  for all (dose-response).

**Table 2.** Plasma t-PA and PAI-1 Antigen and t-PA Activity/Concentrations

Bradykinin Infusion (pmol/min)	Typhoid Vaccination				Placebo Injection			
	0	40	200	1,000	0	40	200	1,000
t-PA antigen (ng/ml)								
Noninfused arm	2.3 ± 0.3	2.7 ± 0.5	2.9 ± 0.5	3.5 ± 0.5	2.8 ± 0.6	3.2 ± 0.8	3.6 ± 0.7	4.4 ± 0.8
Infused arm	3.0 ± 0.5	3.2 ± 0.5	3.9 ± 0.5	6.7 ± 0.7*	2.8 ± 0.6	3.0 ± 0.7	3.8 ± 0.8	6.1 ± 0.9*
Concentration differences between forearms	0.7 ± 0.4	0.5 ± 0.4	1.0 ± 0.6	3.3 ± 0.8§	0.0 ± 0.2	-0.1 ± 0.4	0.2 ± 0.3	1.7 ± 0.5§‡
t-PA activity (IU/ml)								
Noninfused arm	1.3 ± 0.2	1.4 ± 0.2	1.7 ± 0.2	2.6 ± 0.4	1.1 ± 0.2	1.2 ± 0.2	1.5 ± 0.2	2.5 ± 0.5
Infused arm	1.1 ± 0.2	1.6 ± 0.3	3.3 ± 0.6	9.9 ± 1.2*	1.0 ± 0.2	1.3 ± 0.2	2.5 ± 0.5	6.5 ± 1.3*†
Concentration differences between forearms	-0.2 ± 0.1	0.2 ± 0.1	1.6 ± 0.5	7.4 ± 1.0*	-0.1 ± 0.1	0.1 ± 0.1	0.9 ± 0.5	3.9 ± 1.0*‡
PAI antigen (ng/ml)								
Noninfused arm	17 ± 4			16 ± 2	15 ± 2			16 ± 3
Infused arm	18 ± 5			14 ± 2	16 ± 3			15 ± 2
Concentration differences between forearms	1.3 ± 2.4			-1.2 ± 1.3	0.4 ± 0.8			-0.5 ± 1.7

One-way analysis of variance (ANOVA): \*p < 0.001, §p < 0.01 (dose-response). Two-way ANOVA: †p = 0.026, ‡p < 0.01 (vaccination vs placebo); p < 0.001 for all (dose-response).

PAI-1 = plasminogen activator inhibitor type 1; t-PA = tissue-type plasminogen activator.

concentrations did not change during bradykinin infusion (Table 2).

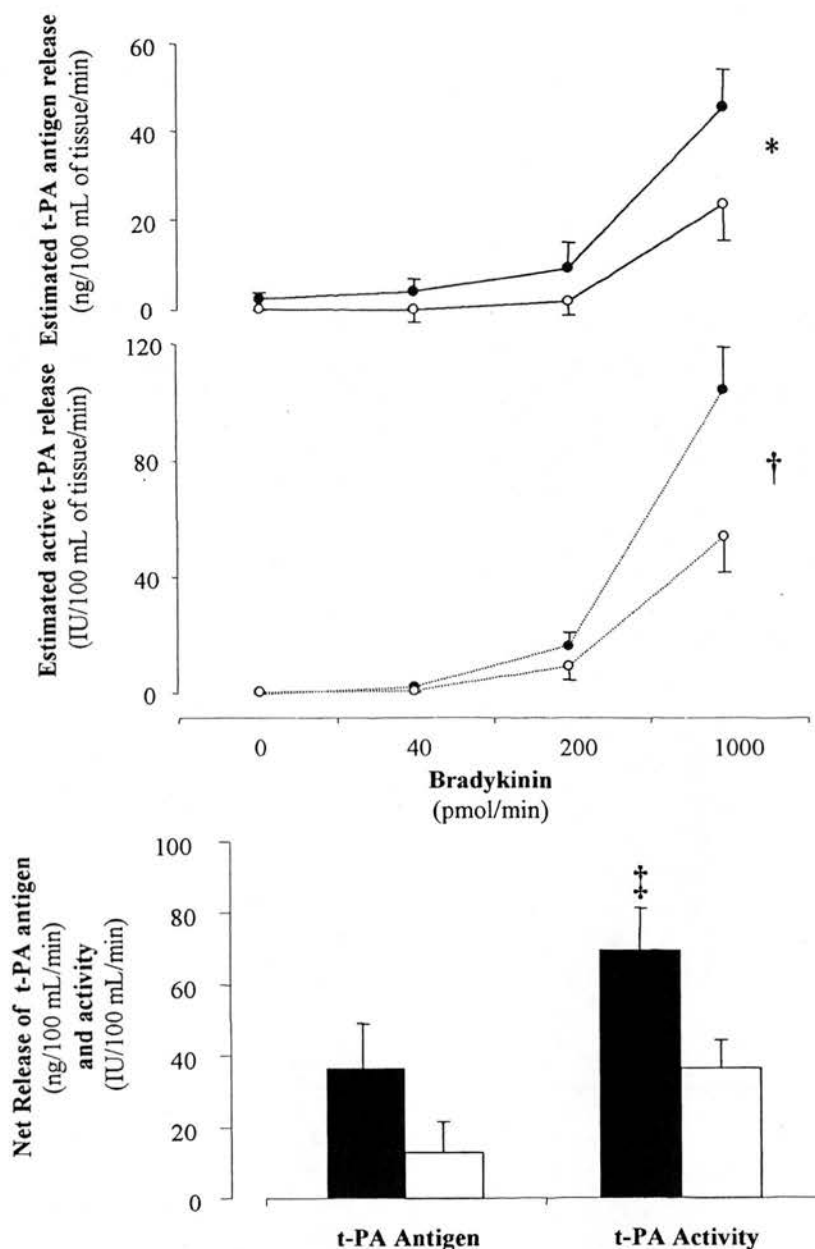
## DISCUSSION

We have demonstrated that an acute mild systemic inflammatory stimulus causes potentiation of bradykinin-induced t-PA release. We conclude that acute systemic inflammation enhances local endothelial t-PA release in men. This may reflect an adaptive mechanism of the vascular endothelium to augment its fibrinolytic response under circumstances of acute inflammation.

**Endothelium-dependent vasomotion.** We assessed basal and stimulated FBF after intra-arterial infusions of the endothelium-dependent vasodilators bradykinin and acetylcholine, and the endothelium-independent vasodilator sodium nitroprusside 6 to 8 h after vaccination. Bradykinin and acetylcholine have both been widely used to investigate the function of vascular endothelium. Impaired arterial vasodilatory response to endothelium-dependent agonists has been shown in patients with hypertension (24), diabetes mellitus (25), and hypercholesterolemia (26). Hingorani et al. (22) have previously reported that *S. typhi* vaccinations generated an inflammatory response that was associated with a temporary suppression of endothelium-dependent vasodilation in the forearm circulation of six healthy volunteers. Although we applied a similar protocol to their study and included overlapping doses of both bradykinin and acetylcholine, we did not replicate their findings of impaired forearm endothelium-dependent vasodilation after vaccination at 6 to 8 h (Fig. 1). This discrepancy may be partly explained by the variability in vasodilatory response with acetylcholine (27) and the higher maximal vasodilator dose used in our studies, although we used a larger sample size and a double-blind, randomized, placebo-controlled, crossover trial.

**Endogenous fibrinolysis.** Tissue plasminogen activator, the key enzyme in the initiation of fibrinolysis, is synthesized in endothelial cells and stored in small, dense vesicles. It is secreted both basally and in response to thrombin and several vasoactive agents through a calcium-dependent and G protein-coupled pathway (28). The regulated endogenous release of t-PA plays a major role in the defense against intravascular thrombosis, especially in the coronary circulation (18). Bradykinin is a vasoactive peptide and potent stimulant for the acute release of t-PA from the vasculature (23,29-31) and is produced locally through activation of the kallikrein-kinin system on the surface of endothelial cells (32). In the present study, bradykinin-induced t-PA antigen and activity release were augmented two to threefold after typhoid vaccination in the absence of systemic hemodynamic effects (Fig. 2).

The underlying mechanisms for our findings are unknown. Inflammation is recognized to induce a protective response towards tissue injury, and it functions as part of normal host surveillance mechanisms. Various compounds associated with the inflammatory response, including histamine, thrombin and endotoxin, have been shown to increase cellular t-PA transcription and expression (33,34). However, we did not observe an increase in the basal plasma concentrations of either t-PA or PAI-1. The inflammatory stimulus, therefore, appears to augment specifically the storage or acute release of t-PA rather than a generalized upregulation of protein synthesis and basal secretion. This may be mediated by pro-inflammatory cytokines, such as IL-6, that modulate cellular activation, leading to alterations in endothelial function. In particular, molecular and pharmacologic evidence supports the role of bradykinin B2 receptors in the acute phase of inflammation, and upregulation of B2 receptors may account for the potentiation of bradykinin-induced t-PA release.



**Figure 2.** Estimated net release of tissue-type plasminogen activator (t-PA) antigen (solid line) and activity (dotted line) in subjects who were administered typhoid vaccination (closed circles and bars) and saline placebo (open circles and bars). Lower panel represents area under the curve for the response. Analysis of variance, \* $p < 0.05$ ; † $p = 0.006$  (vaccination vs. placebo);  $t$  test, ‡ $p < 0.05$  (vaccination vs. placebo).

**Clinical implications.** The augmentation of acute t-PA release after typhoid vaccination suggests that mild acute inflammation may induce antithrombotic properties in the forearm circulation. This may represent an adaptive response to inhibit intravascular thrombus deposition under circumstances of acute vascular inflammation. This observation is consistent with the increase in endogenous fibrinolysis in systemic inflammation induced by experimental endotoxemia in healthy subjects (35). However, in susceptible individuals, such as those with ischemic heart disease and chronic inflammation, this adaptive and protective acute response may fail or become depleted, leading to

thrombus propagation and vessel occlusion. The fibrinolytic response to acute systemic inflammation in patients with ischemic heart disease and the influence of anti-inflammatory therapies, such as aspirin, now needs to be established. Indeed, recent evidence has suggested that preventative treatment with aspirin is able to reverse inflammation-induced endothelial dysfunction (36).

Epidemiologic studies have demonstrated an association between the risk of future cardiovascular events and both plasma inflammatory markers (11–13) and fibrinolytic factors (37). Therefore, the current observations are consistent with the suggestion that elevated plasma t-PA concentra-

tions may provide a marker of vascular inflammation. Irrespective of whether these common associations are partly or wholly explained by inflammation-induced t-PA release, understanding the regulation of both acute and chronic t-PA release will have important clinical implications and may help to develop more effective strategies in the management of atherosclerotic disease.

**Study limitations.** There are some limitations to our study. We administered the typhoid vaccination in the deltoid muscle of each subject's dominant arm rather than the gluteus muscle. However, because blood flow was assessed in the forearm and intra-arterial vasodilators were administered in the contralateral nondominant arm, it would be highly unlikely that the site of vaccination would have affected the response in the infused forearm. It would also have been preferable to assess the vascular responses immediately before and 6 to 8 h after vaccination on the same day. However, this would require repeated arterial cannulations within the same day, and we have previously demonstrated that endothelium-dependent vasodilation and t-PA release is reproducible when performed at least one week apart (38,39). The study subjects were healthy and young, and we acknowledge that the response in older subjects may be quite different. Finally, we studied peripheral vascular function; therefore, these findings may not be directly applicable to other vascular beds. However, endothelial dysfunction is often a generalized process, and we have previously shown (21,40) consistent endogenous fibrinolytic responses between the forearm and coronary circulation.

**Conclusions.** We have demonstrated that mild inflammation generated by typhoid vaccination results in a significant potentiation of bradykinin-induced t-PA release from the vascular endothelium. Additional studies are now required to determine the underlying mechanism and to assess the effects of acute and chronic inflammation on endogenous fibrinolysis in health and disease.

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## **PUBLICATION 10**

# Intra-Arterial Tumor Necrosis Factor- $\alpha$ Impairs Endothelium-Dependent Vasodilatation and Stimulates Local Tissue Plasminogen Activator Release in Humans

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Keith A.A. Fox, David E. Newby

**Objective**—Inflammation contributes to the pathogenesis of cardiovascular disease, potentially through the actions of proinflammatory cytokines. We assessed the direct effects of local intra-arterial tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-6, and endotoxin on blood flow and endogenous tissue plasminogen activator (t-PA) release in vivo in humans.

**Methods and Results**—In a double-blind, randomized, placebo-controlled trial, blood flow, plasma cytokine, and fibrinolytic parameters were measured using venous occlusion plethysmography and blood sampling. Ten subjects received intrabrachial TNF- $\alpha$ , interleukin-6, and endotoxin infusions, and 8 additional subjects received intrabrachial infusions of bradykinin, acetylcholine, and sodium nitroprusside after pretreatment with TNF- $\alpha$ . TNF- $\alpha$  but not interleukin-6, endotoxin, or placebo caused a gradual and sustained  $\approx 20$ -fold increase in plasma t-PA concentrations ( $P < 0.001$ ) that was associated with elevated plasma interleukin-6 concentrations ( $P < 0.05$ ) but without an effect on blood flow or plasminogen activator inhibitor type 1 antigen. Compared with placebo, TNF- $\alpha$  pretreatment impaired bradykinin- and acetylcholine-induced vasodilatation ( $P < 0.03$ ) and resulted in a doubling of bradykinin-induced t-PA release ( $P < 0.05$ ).

**Conclusions**—Intra-arterial TNF- $\alpha$  causes an acute local vascular inflammation that is associated with impaired endothelium-dependent vasomotion as well as a sustained and substantial increase in endothelial t-PA release. TNF- $\alpha$  has potentially both adverse vasomotor and beneficial profibrinolytic effects on endothelial function. (*Arterioscler Thromb Vasc Biol.* 2003;23:695-701.)

**Key Words:** cytokines ■ endothelium ■ fibrinolysis ■ inflammation ■ vasodilatation

There is emerging evidence that systemic inflammation plays a major role in the pathogenesis of cardiovascular disease. The proinflammatory cytokines, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-6 (IL-6) in particular, have been implicated in the initiation and maintenance of systemic and vascular inflammation associated with atherosclerosis and coronary artery disease. Indeed, plasma concentrations of these cytokines are elevated in patients with ischemic heart disease<sup>1,2</sup> and have been shown to predict the future risk of myocardial infarction in apparently healthy individuals.<sup>3</sup>

The vascular endothelium plays a vital role in the control of blood flow, hemostasis, fibrinolysis, and inflammation, and changes in endothelial function may underlie the association between inflammation and the risk of cardiovascular disease.

Tissue plasminogen activator (t-PA) is a fibrinolytic factor released from the endothelium and lyses intravascular fibrin.<sup>4</sup> If endogenous fibrinolysis is to be effective, then the rapid mobilization of t-PA from the endothelium is essential,

because thrombus dissolution is much more effective if t-PA is incorporated during, rather than after, thrombus formation.<sup>5</sup> However, in the presence of proinflammatory states or an imbalance in endogenous fibrinolysis, intravascular thrombus may propagate, ultimately leading to arterial occlusion and tissue infarction.<sup>6</sup>

TNF- $\alpha$  and endotoxin have been reported to induce local vascular inflammation and impair endothelium-dependent vasodilatation in the venous circulation of humans.<sup>7</sup> Although mild systemic inflammation has also been shown to alter endothelial function,<sup>8</sup> the underlying mechanisms for these observations have not been elucidated, and the direct in vivo effects of cytokines and inflammatory stimuli on local arterial endothelial vasomotor and fibrinolytic function are unknown.

The aims of this study were to investigate the acute effects of local intra-arterial inflammatory cytokines (IL-6 and TNF- $\alpha$ ) and bacterial endotoxin (lipopolysaccharide [LPS]) exposure on vasomotor function and endothelial t-PA release in vivo in humans.

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## Methods

### Subjects

Eighteen healthy nonsmoking men 21 to 25 years of age participated in the study, which was undertaken with the approval of the local research ethics committee in accordance with the Declaration of Helsinki and the written informed consent of each subject. None of the subjects had infective illnesses or received medication in the week before study. All subjects abstained from alcohol for 24 hours and from food and caffeine-containing drinks for at least 4 hours before each study. All studies were performed in a quiet, temperature-controlled room.

### Cytokines and Drugs

TNF- $\alpha$  (Knoll Pharmaceuticals),<sup>9</sup> IL-6 (Novartis Pharma AG),<sup>10</sup> LPS (lot G-1, USPC1),<sup>11</sup> bradykinin (Clinalfa), acetylcholine (Cibavision Ophthalmics), and sodium nitroprusside (David Bull Laboratories) were administered after dissolution in 0.9% saline. TNF- $\alpha$ , IL-6, and LPS were prepared as stock solutions and stored at  $-80^{\circ}\text{C}$  in aliquots. All other drugs were freshly prepared on the study day.

### Hemodynamic Measurements

Blood flow was measured in both forearms by venous occlusion plethysmography using mercury-in-silastic strain gauges, as previously described.<sup>12</sup> Blood pressure was monitored in the noninfused arm at intervals throughout each study with a semiautomated noninvasive oscillometric sphygmomanometer.

### Venous Sampling and Assays

Venous blood (10 mL) was withdrawn simultaneously from each arm and collected into tubes containing acidified buffered citrate (for t-PA assays), trisodium citrate (for plasminogen activator inhibitor type 1 [PAI-1] assays), and potassium ethylene diamine tetraacetic acid (EDTA) (for cytokine assays). Citrate and acidified buffered citrate samples were centrifuged at 2000g for 30 minutes at  $4^{\circ}\text{C}$  and EDTA samples at 1000g for 10 minutes at  $20^{\circ}\text{C}$ . Platelet-free plasma was decanted and stored at  $-80^{\circ}\text{C}$  before assay. Plasma t-PA, PAI-1, TNF- $\alpha$ , IL-6, and von Willebrand factor (vWF) antigen concentrations were determined as previously described<sup>13,14</sup> using enzyme-linked immunosorbent assays (Coaliza t-PA and PAI-1, Chromogenix AB; Quantikine human TNF and IL-6 immunoassays, R&D Systems; and Dako A/S, respectively) and fibrinolytic activities using a photometric method (Coatest t-PA and PAI-1, Chromogenix AB). Hematocrit and white cell count were determined using an automated Coulter counter (Beckman Coulter ACT.8).

### Study Design

Subjects rested recumbent, strain gauges and cuffs were applied, and venous cannulae (17-G) were inserted into both antecubital fossae. The brachial artery of the nondominant arm was cannulated with a 27-SWG needle under local anesthesia. The total rate of intra-arterial infusion was maintained constant throughout at 1 mL/min. Forearm blood flow was measured every 6 to 10 minutes.

### Cytokine and Endotoxin Administration

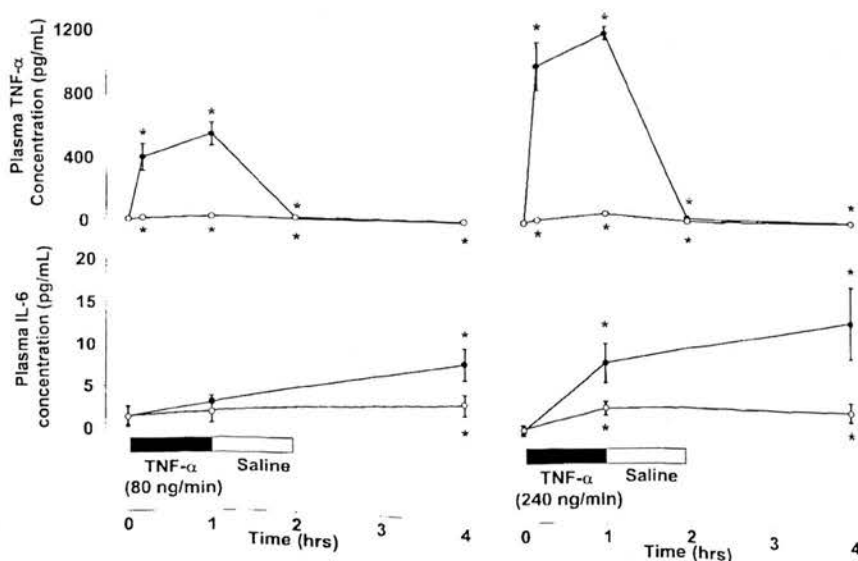
In a randomized, double-blind study, 10 subjects attended on 3 occasions at least 1 week apart, and saline was infused for 30 minutes to allow time for equilibration. Subjects were then randomized to receive intra-arterial infusions of low-dose TNF- $\alpha$  (80 ng/min;  $n=6$ ), high-dose TNF- $\alpha$  (240 ng/min;  $n=6$ ), IL-6 (30 ng/min;  $n=6$ ), LPS (100 pg/min;  $n=6$ ), or saline placebo (1 mL/min;  $n=6$ ) over 60 minutes. This was followed by an additional 60-minute saline washout infusion. Venous samples were obtained at baseline, 10, 20, 40, and 60 minutes during drug infusion, and 30, 60, and 180 minutes after cessation of drug infusion. Cytokine and LPS doses were chosen to achieve local concentrations comparable with healthy volunteer studies<sup>7,11,15</sup> and those seen in cardiovascular disease.<sup>1,16</sup>

### Effect of Tumor Necrosis Factor- $\alpha$ on Endothelial Function

Eight subjects attended on 2 occasions at least 1 week apart in a randomized, double-blind, placebo-controlled, crossover trial. They received an intra-arterial infusion of either TNF- $\alpha$  (80 ng/min) or saline placebo (1 mL/min) over 60 minutes. After an additional 60 minutes of saline infusion, intra-arterial bradykinin was administered at 100, 300, and 1000 pmol/min for 10 minutes at each dose, acetylcholine at 5, 10, and 20  $\mu\text{g}/\text{min}$ , and sodium nitroprusside at 2, 4, and 8  $\mu\text{g}/\text{min}$  for 5 minutes at each dose. Infusions of the vasoactive drugs were separated by 15-minute infusions of saline. Venous samples were obtained at baseline, after 60 minutes of TNF- $\alpha$ /placebo infusion, before and during each dose of bradykinin, and 15 minutes after cessation of bradykinin infusion. Venous samples were not obtained during acetylcholine or sodium nitroprusside infusion, because they do not affect plasma t-PA or PAI-1 concentrations in this forearm model.<sup>12,17,18</sup>

### Data Analysis and Statistics

Plethysmographic data were extracted from the chart data files, and the last 5 linear recordings in each measurement period were averaged. Estimated net t-PA antigen and activity release was defined previously<sup>12-14</sup> as the product of the infused forearm plasma flow and the concentration difference between the infused and noninfused forearms. Because basal t-PA concentrations were al-



**Figure 1.** Plasma concentrations of TNF- $\alpha$  (top) and IL-6 (bottom) in the infused (●) and noninfused (○) arms after 1 hour of intra-arterial infusion of TNF- $\alpha$  at 80 ng/min (left) and 240 ng/min (right).  $P \leq 0.01$  (ANOVA) for all responses except IL-6 concentrations in the noninfused arm.  $P < 0.05$  (ANOVA, infused vs noninfused arm) for all responses. \* $P < 0.05$  (paired  $t$  test; vs baseline).

**TABLE 1. Forearm Blood Flow (mL/100 mL/min) During TNF- $\alpha$ , IL-6, LPS, and Saline Placebo Infusion**

	TNF- $\alpha$ , 80 ng/min	TNF- $\alpha$ , 240 ng/min	IL-6, 30 ng/min	LPS, 100 pg/min	Saline
<b>Infused arm</b>					
Baseline	2.2 $\pm$ 0.2	2.6 $\pm$ 0.6	2.4 $\pm$ 0.1	2.5 $\pm$ 0.3	2.2 $\pm$ 0.3
1 hour	1.9 $\pm$ 0.3	2.4 $\pm$ 0.9	1.9 $\pm$ 0.2	2.3 $\pm$ 0.3	2.0 $\pm$ 0.3
2 hours	1.8 $\pm$ 0.3	2.1 $\pm$ 0.7	1.8 $\pm$ 0.2	2.0 $\pm$ 0.3	1.7 $\pm$ 0.3
<b>Noninfused arm</b>					
Baseline	2.2 $\pm$ 0.4	2.6 $\pm$ 0.5	2.0 $\pm$ 0.2	2.3 $\pm$ 0.4	1.8 $\pm$ 0.2
1 hour	2.6 $\pm$ 0.5	2.6 $\pm$ 0.8	2.0 $\pm$ 0.3	2.0 $\pm$ 0.3	1.7 $\pm$ 0.2
2 hours	2.9 $\pm$ 0.7	2.4 $\pm$ 0.5	1.8 $\pm$ 0.2	1.8 $\pm$ 0.2	1.9 $\pm$ 0.3

Values are mean $\pm$ SEM.

tered by pretreatment with TNF- $\alpha$ , t-PA antigen and activity release during bradykinin infusion was calculated by subtracting the mean t-PA release before and 15 minutes after cessation of bradykinin infusion.

Data were examined, where appropriate, by ANOVA with repeated measures and 2-tailed paired Student's *t* test using Statview (SAS Institute, Inc). All results are expressed as mean $\pm$ SEM. Statistical significance was assigned at the 5% level.

## Results

All subjects remained well throughout the study and reported no adverse effects. There were no effects on hematocrit, body temperature, or white cell count throughout all studies (data on file).

### Cytokine and Endotoxin Administration

#### Cytokine Assays

Plasma TNF- $\alpha$  concentrations increased from 1 $\pm$ 0 and 2 $\pm$ 1 pg/mL to 539 $\pm$ 71 and 1164 $\pm$ 41 pg/mL in the infused arm ( $P<0.001$  for both) and to 20 $\pm$ 2 and 62 $\pm$ 8 pg/mL in the noninfused arm ( $P<0.001$  for both) during 80 and 240 ng/min of TNF- $\alpha$ , respectively (Figure 1). This was accompanied by a gradual increase in plasma IL-6 concentrations (Figure 1). In the infused arm, IL-6 infusion increased plasma IL-6 concentrations from 2 $\pm$ 1 to 14 $\pm$ 3 pg/mL ( $P=0.01$ ) and

LPS infusion increased plasma TNF- $\alpha$  and IL-6 concentrations from 1 $\pm$ 3 to 7 $\pm$ 1 pg/mL ( $P=0.01$ ) and from 1 $\pm$ 0 to 6 $\pm$ 2 pg/mL ( $P=0.02$ ), respectively.

#### Hemodynamic Effects

Intra-arterial saline placebo, TNF- $\alpha$ , IL-6, and LPS infusions had no effect on heart rate, blood pressure, or forearm blood flow throughout all studies (data on file; Table 1).

#### Fibrinolytic and Hemostatic Assays

There were no changes in plasma t-PA antigen concentrations during IL-6, LPS, or saline placebo infusions (data on file). Plasma t-PA antigen and activity concentrations increased in the infused arm by up to 20-fold after both low- and high-dose TNF- $\alpha$  infusions ( $P<0.001$ ; Figure 2). Plasma t-PA concentrations increased slowly, being detectable at 20 minutes and peaking at 60 minutes of infusion. Thereafter, plasma t-PA concentrations fell but remained elevated 4 hours after initiation of the infusion, with an apparently stable elevation between 2 and 4 hours.

Plasma PAI-1 and vWF antigen concentrations were unchanged throughout all studies, although plasma PAI-1 activity was reduced in the infused arm only after high-dose TNF- $\alpha$  infusion ( $P=0.003$ ; Table 2).

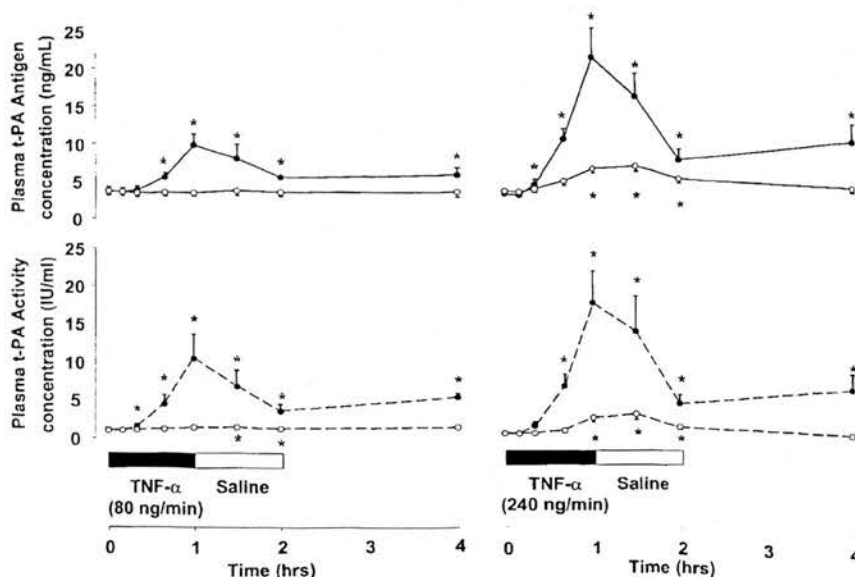
### Effect of Tumor Necrosis Factor- $\alpha$ on Endothelial Function

#### Cytokine Assays

Intra-arterial TNF- $\alpha$  infusion (80 ng/min) increased plasma TNF- $\alpha$  concentrations in the infused and noninfused arm from 2 $\pm$ 1 and 2 $\pm$ 1 pg/mL to 561 $\pm$ 108 and 15 $\pm$ 1 pg/mL, respectively ( $P<0.001$  for both).

#### Hemodynamic Effects

Intra-arterial TNF- $\alpha$  or placebo infusion had no significant effects on heart rate, blood pressure, or basal forearm blood flow up to 2 hours after commencement of the infusion ( $P=NS$  for both). Forearm blood flow increased in a dose-dependent manner during bradykinin, acetylcholine, and so-



**Figure 2.** Plasma concentrations of t-PA antigen (solid lines) and activity (dashed lines) in the infused (●) and noninfused (○) arms after 1 hour of intra-arterial infusion of TNF- $\alpha$  at 80 ng/min (left) and 240 ng/min (right).  $P<0.001$  (ANOVA) for all responses except noninfused arm after TNF- $\alpha$  80 ng/min infusion.  $P<0.001$  (ANOVA, infused vs noninfused arm) for all responses. \* $P<0.05$  (paired *t* test; vs baseline).

**TABLE 2. Plasma PAI-1 Antigen and Activity Concentrations During TNF- $\alpha$  Infusion**

	TNF- $\alpha$ , 80 ng/min		TNF- $\alpha$ , 240 ng/min	
	Infused	Noninfused	Infused	Noninfused
PAI-1 antigen, ng/mL				
0 minutes	17 $\pm$ 4	19 $\pm$ 5	23 $\pm$ 5	24 $\pm$ 5
60 minutes	18 $\pm$ 4	20 $\pm$ 4	25 $\pm$ 6	26 $\pm$ 8
120 minutes	14 $\pm$ 3	16 $\pm$ 3	18 $\pm$ 5	24 $\pm$ 5
240 minutes	15 $\pm$ 4	14 $\pm$ 3	21 $\pm$ 5	36 $\pm$ 17
PAI-1 activity, IU/mL				
0 minutes	12 $\pm$ 2	11 $\pm$ 1	10 $\pm$ 2	9 $\pm$ 2
60 minutes	9 $\pm$ 2	10 $\pm$ 2	5 $\pm$ 2*	12 $\pm$ 3
120 minutes	11 $\pm$ 1	14 $\pm$ 3	6 $\pm$ 1*	10 $\pm$ 2
240 minutes	8 $\pm$ 1	11 $\pm$ 2	4 $\pm$ 1*	12 $\pm$ 1

Values are mean $\pm$ SEM.\* $P=0.003$ , ANOVA, infused vs noninfused arm.

dium nitroprusside infusions ( $P\leq 0.02$ ; Figure 3). Compared with placebo, TNF- $\alpha$  infusion caused impaired endothelium-dependent vasodilatation to bradykinin ( $P=0.029$ ) and acetylcholine ( $P=0.01$ ) but had no effect on endothelium-independent vasodilatation to sodium nitroprusside ( $P=0.43$ ; Figure 3).

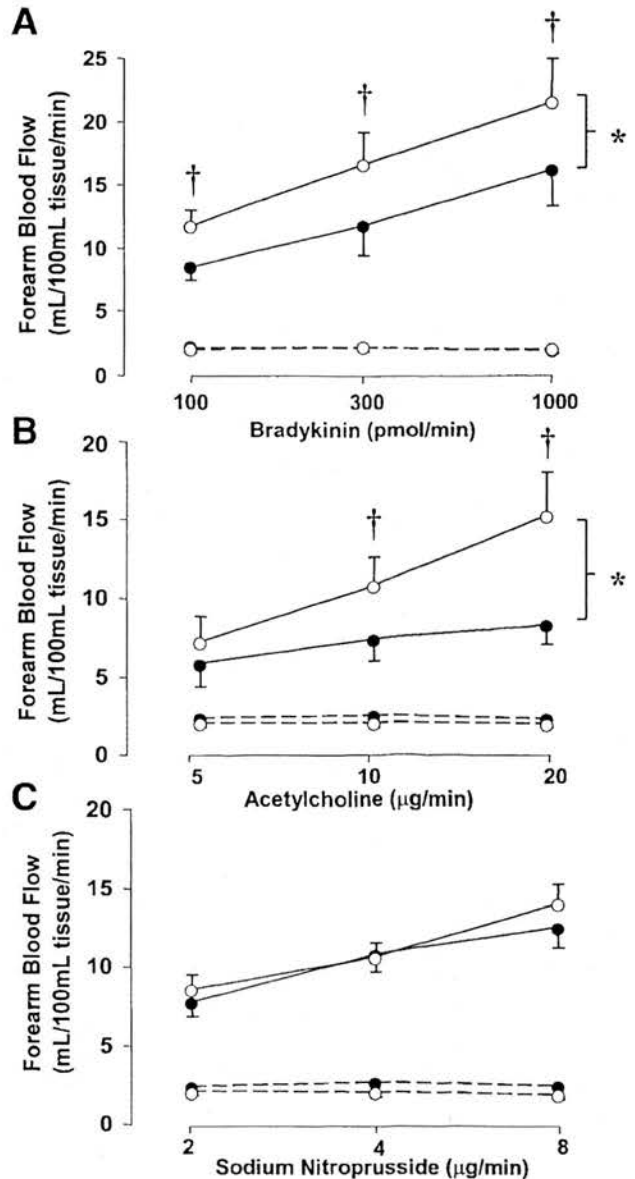
#### Fibrinolytic Assays

Plasma t-PA antigen and activity concentrations increased from  $3.5\pm 0.6$  to  $8.5\pm 1.6$  ng/mL ( $P=0.004$ ) and from  $1.1\pm 0.3$  to  $9.2\pm 1.9$  IU/mL ( $P=0.003$ ), respectively, after 60 minutes of TNF- $\alpha$  infusion. Again, this rise was sustained with an apparently stable elevation between 2 and 3 hours, ie, 1 to 2 hours after cessation of TNF infusion (Figure 4). Plasma t-PA antigen and activity concentrations were unchanged in the noninfused arm and during placebo infusion.

Plasma t-PA antigen and activity concentrations increased in a dose-dependent manner during bradykinin infusion ( $P\leq 0.01$ ). Compared with placebo, pretreatment with TNF- $\alpha$  resulted in higher plasma t-PA antigen and activity concentrations during bradykinin infusion ( $11.3\pm 0.8$  versus  $6.8\pm 0.5$  ng/mL and  $16.5\pm 3.9$  versus  $6.6\pm 2.0$  IU/mL at peak bradykinin dose;  $P<0.002$ ) (Figure 4) and a marked augmentation in the estimated net release of t-PA antigen and activity ( $P=0.018$  and  $P=0.037$ , respectively; Figure 5). For the 30-minute bradykinin dose-response infusion, pretreatment with TNF- $\alpha$  resulted in 83% and 132% increases in area under the curve for t-PA antigen and activity release, respectively ( $P\leq 0.05$ ).

#### Discussion

We have demonstrated for the first time that local intra-arterial TNF- $\alpha$  administration can be used to generate an *in vivo* local vascular inflammatory model in humans that is associated with endothelial dysfunction and a unique profile of endogenous t-PA release. In contrast to IL-6 and LPS, TNF- $\alpha$  causes a slow-onset, sustained, and selective release of t-PA from the vascular endothelium in the absence of basal vasomotor effects. Moreover, pretreatment with TNF- $\alpha$  attenuates endothelium-dependent vasodilatation but augments



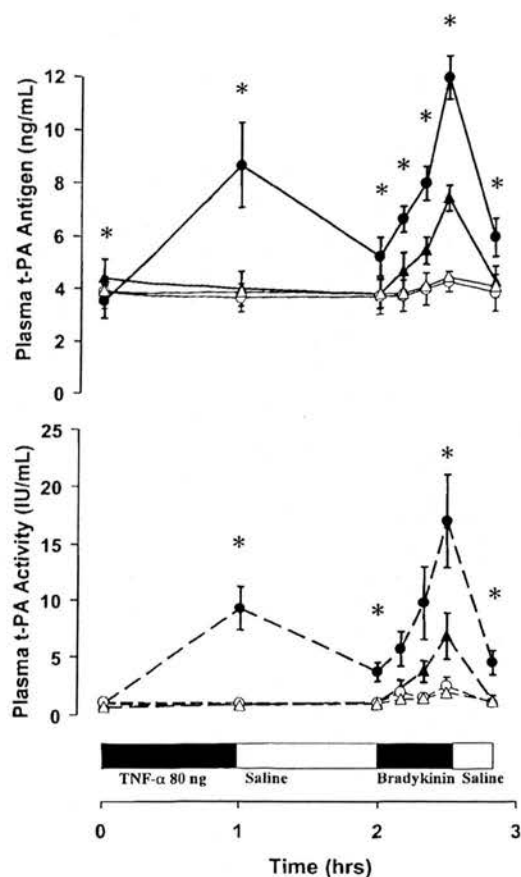
**Figure 3.** Infused (solid line) and noninfused (dashed line) forearm blood flow responses to incremental doses of bradykinin (a), acetylcholine (b), and sodium nitroprusside (c) in subjects who were pretreated with TNF- $\alpha$  (●) or saline placebo (○).  $P\leq 0.02$  (ANOVA) for all infused arm responses. \* $P<0.03$  (ANOVA, TNF- $\alpha$  vs saline placebo).

acute bradykinin-induced t-PA release. These findings indicate that TNF- $\alpha$  and acute vascular inflammation have complex effects on endothelial function. Although the profibrinolytic actions may reflect a protective mechanism in acute inflammation, TNF- $\alpha$  also directly impairs endothelium-dependent vasomotor responses.

#### Model of Local Vascular Inflammation

We have here developed a model of local vascular inflammation *in vivo* in humans. Using unilateral intrabrachial infusions, we achieved high local plasma TNF- $\alpha$  concentrations that were comparable to the plasma concentrations seen



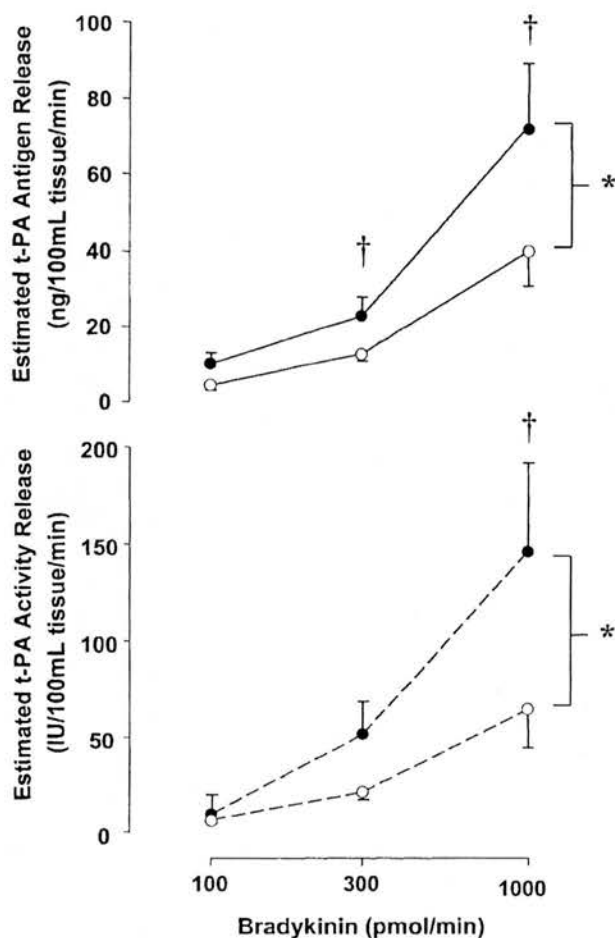


**Figure 4.** Plasma concentrations of t-PA antigen (solid lines) and activity (dashed lines) in infused ( $\bullet$ ) and noninfused ( $\circ$ ) arms after 1 hour of intra-arterial infusion of TNF- $\alpha$  (circles) or saline placebo (triangles) and during bradykinin infusion.  $P < 0.001$  (ANOVA) for all infused arm responses.  $P < 0.001$  (ANOVA, infused versus noninfused arm) for all responses.  $P < 0.001$  (ANOVA, TNF- $\alpha$  vs saline placebo) for infused arm responses. \* $P < 0.05$  (paired  $t$  test; TNF- $\alpha$  vs placebo) for infused arm responses.

in patients with severe heart failure.<sup>16</sup> A direct local vascular and endothelial inflammatory response was confirmed by the local rise in plasma IL-6 and t-PA concentrations. However, the fibrinolytic effects of TNF- $\alpha$  were not mediated through IL-6 release, because isolated IL-6 infusion had no effect on t-PA release. Indeed, in pilot studies, we found that high-dose intrabrachial IL-6 infusion sufficient to increase plasma IL-6 concentrations to  $>100$  pg/mL have also failed to produce significant effects on forearm blood flow or t-PA release.

#### Effects of TNF- $\alpha$ on t-PA Release

The profile of t-PA release during local intra-arterial TNF- $\alpha$  administration is unique and has not been previously described in vivo in humans. Previous studies in healthy volunteers have reported changes in plasma fibrinolytic<sup>15</sup> and coagulation<sup>20</sup> factors during systemic TNF- $\alpha$  administration. TNF- $\alpha$  has pleiotropic effects and may cause these effects through actions on specific tissues or via secondary mediators released from organs such as the liver. In the present study, we have assessed local peripheral vascular responses to direct



**Figure 5.** Estimated net t-PA antigen (solid lines) and activity (dashed lines) release during bradykinin infusion corrected for baseline t-PA concentrations in subjects who were pretreated with TNF- $\alpha$  ( $\bullet$ ) or saline placebo ( $\circ$ ).  $P < 0.001$  (ANOVA) for all responses. \* $P < 0.05$  (ANOVA, TNF- $\alpha$  vs saline placebo). † $P \leq 0.05$  (paired  $t$  test; TNF- $\alpha$  vs saline placebo).

intra-arterial TNF- $\alpha$  and have shown that it causes selective endothelial t-PA release in the forearm without demonstrable effects on plasma vWF or PAI-1 antigen concentrations. Although there was a modest rise in the IL-6 and t-PA concentrations in the noninfused arm, this may represent overspill from the infused arm, where the concentrations increased by up to 20-fold. In contrast to previous systemic studies,<sup>15,20</sup> subjects here remained asymptomatic and there was no associated pyrexia, consistent with the absence of a major systemic response.

We<sup>12</sup> and others<sup>17,18</sup> have previously reported acute rapid t-PA release during intra-arterial substance P, bradykinin, and methacholine infusions. Using these agents, there is a near-instantaneous onset and offset of action with no sustained increase in t-PA release after cessation of administration. Moreover, there is always an associated change in vascular tone and regional blood flow, because these agents also cause vasodilatation. In contrast, TNF- $\alpha$  administration had no effect on basal blood flow and caused a slow onset and sustained release of t-PA that was not apparent until 20

minutes after commencement of the infusion and continued for at least 3 hours after its cessation.

Endothelial cells synthesize and secrete t-PA both constitutively and facultatively. The facultative release of t-PA occurs in response to stimulation by several physiological agonists, including thrombin and bradykinin.<sup>19</sup> This large and rapid release arises from the translocation of a dynamic intracellular storage pool of t-PA.<sup>4,19</sup> Agonists such as bradykinin are likely to stimulate t-PA release via exocytosis of these granules because of the near-instantaneous release of t-PA and the *ex vivo* animal evidence that inhibition of protein synthesis by cycloheximide has no effect on bradykinin-induced acute t-PA release.<sup>19</sup> The profile of t-PA release seen with TNF- $\alpha$  is distinct from this pathway. Although the mechanism has not been elucidated in our study, we speculate that it may arise from an increase in *de novo* t-PA synthesis and its constitutive release rather than through the previously described facultative pathways. However, the initial detectable rise in t-PA release seen at 20 minutes may be too early for protein synthesis to occur. Other potential mechanisms such as activation of adherent leukocytes may cause the generation of secondary mediators that enhance t-PA release.

Infusion of TNF- $\alpha$  seems to cause an isolated increase in local t-PA release without affecting other endothelium-derived fibrinolytic or hemostatic factors, namely PAI-1 and vWF, respectively. This suggests a selective and specific action of TNF- $\alpha$  on the endothelium and forearm vascular bed. The fall in plasma PAI-1 activity seen in the infused arm during high-dose TNF- $\alpha$  infusion is likely to reflect the magnitude of the local t-PA released and its immediate binding with PAI-1. Although other investigators have reported elevated plasma PAI-1 antigen concentrations within 3 hours of systemic administration of TNF- $\alpha$  and endotoxin,<sup>15,21</sup> we did not detect any changes in PAI-1 antigen concentrations. This suggests that cytokine-induced increases in PAI-1 release are mediated through a systemic mechanism, such as hepatic or adipocyte synthesis, or produced in response to markedly elevated systemic plasma t-PA concentrations.

### Effects of TNF- $\alpha$ on Endothelium-Dependent Vasomotion

Bhagat and Vallance<sup>7</sup> have previously shown that TNF- $\alpha$  directly induced endothelial dysfunction in the venous circulation of healthy volunteers. We have extended these findings and demonstrated that TNF- $\alpha$  also impairs resistance vessel endothelium-dependent vasodilatation, possibly through the development of acute arterial endothelial injury. The effects of TNF- $\alpha$  were specific for the endothelium, because endothelium-independent vasodilatation to the nitric oxide donor, sodium nitroprusside, was unaltered.

TNF- $\alpha$  may alter endothelial vasomotor responses through various mechanisms including decreased constitutive nitric oxide synthase expression,<sup>22</sup> increased inducible nitric oxide synthase expression,<sup>22</sup> and enhanced production of reactive oxygen species.<sup>23</sup> Additional studies are needed to clarify the precise mechanism, although it is tempting to speculate that TNF- $\alpha$  may, in part, be responsible for inducing endothelial

dysfunction in cardiovascular conditions associated with inflammation.

### Effects of TNF- $\alpha$ on Acute Endogenous Fibrinolysis

We have additionally demonstrated that TNF- $\alpha$  potentiates bradykinin-induced endothelial t-PA release despite reduced endothelium-dependent vasodilatation. This suggests that acute local vascular inflammation induces antithrombotic properties that may represent an adaptive response to inhibit intravascular thrombus deposition at sites of vascular injury. In particular, molecular and pharmacological evidence supports the role of bradykinin B2 receptors in the acute phase of inflammation, and upregulation of B2 receptors by TNF- $\alpha$  may account for the observed profibrinolytic effect. The augmented bradykinin response is also likely to reflect the proposed increase in *de novo* t-PA production induced by TNF- $\alpha$  pretreatment.

### Clinical Implications

There is a consistent link between endothelial dysfunction and cardiovascular disease, with impaired endothelium-dependent vasodilatation having been described in atherosclerotic conditions<sup>24</sup> and its associated risk factors, such as hypercholesterolemia.<sup>25</sup> The major findings of our study will be particularly pertinent to cardiovascular conditions, such as acute coronary syndromes and congestive heart failure, in which inflammation and impaired endothelium-dependent vasodilatation occur.

We have found that although TNF- $\alpha$  adversely affects certain aspects of endothelial function, such as endothelium-dependent vasodilatation, it enhances other protective mechanisms, such as the endogenous fibrinolytic capacity. This reflects the complex and pleiotropic nature of TNF- $\alpha$ , which functions as part of the normal host surveillance mechanisms and responses to tissue injury. These diverse effects may explain some of the contradictory findings of recent clinical studies. For example, in patients with heart failure, TNF- $\alpha$  antagonism causes marked improvement in endothelium-dependent vasodilatation<sup>26</sup> but has failed to demonstrate clinical benefit in the RECOVER and RENAISSANCE randomized controlled trials.<sup>27</sup> Thus, the benefits of restoring endothelium dependent vasomotor function by TNF- $\alpha$  antagonism may be counterbalanced by inhibiting other potentially beneficial effects such as enhancing acute endogenous t-PA release.

### Conclusions

This is the first study to delineate the direct effects of intra-arterial TNF- $\alpha$  administration on local vascular tone and endogenous fibrinolysis *in vivo* in humans. It supports the crucial role of TNF- $\alpha$  in cardiovascular disease and provides evidence for its direct and pleiotropic effects on the circulation and endogenous fibrinolysis. Our findings have particular implications for the future development of effective anticytokine and anti inflammatory strategies in cardiovascular disease.

### Acknowledgments

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## **SECTION 2**

### **CARDIOVASCULAR RISK FACTORS (Publications 11-16)**

**PUBLICATION 11**



## Endothelial Dysfunction, Impaired Endogenous Fibrinolysis, and Cigarette Smoking

### A Mechanism for Arterial Thrombosis and Myocardial Infarction

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**Background**—Effective endogenous fibrinolysis requires rapid release of tissue plasminogen activator (tPA) from the vascular endothelium. Smoking is a known risk factor for arterial thrombosis and myocardial infarction, and it causes endothelial dysfunction. We therefore examined the effects of cigarette smoking on substance P-induced tPA release in vivo in humans.

**Methods and Results**—Blood flow and plasma fibrinolytic factors were measured in both forearms of 12 smokers and 12 age- and sex-matched nonsmokers who received unilateral brachial artery infusions of substance P (2 to 8 pmol/min). In both smokers and nonsmokers, substance P caused dose-dependent increases in blood flow and local release of plasma tPA antigen and activity ( $P < 0.001$  for all) but had no effect on the local release of plasminogen activator inhibitor type 1. Compared with nonsmokers, increases in forearm blood flow ( $P = 0.03$ ) and release of tPA antigen ( $P = 0.04$ ) and activity ( $P < 0.001$ ) caused by substance P were reduced in smokers. The area under the curve for release of tPA antigen and activity decreased by 51% and 53%, respectively.

**Conclusions**—Cigarette smoking causes marked inhibition of substance P-induced tPA release in vivo in humans. This provides an important mechanism whereby endothelial dysfunction may increase the risk of atherothrombosis through a reduction in the acute fibrinolytic capacity. (*Circulation*. 1999;99:1411-1415.)

**Key Words:** plasminogen activators ■ endothelium ■ endothelium-derived factors ■ blood flow

Acute rupture of a coronary atheromatous plaque and subsequent coronary artery thrombosis causes the majority of sudden cardiac deaths and myocardial infarctions.<sup>1,2</sup> Cigarette smoking not only is strongly associated with atherosclerosis<sup>3</sup> and ischemic heart disease<sup>4</sup> but also is a major risk factor for acute coronary thrombosis.<sup>1,5</sup> Indeed, 75% of sudden cardiac deaths due to acute thrombosis are in cigarette smokers.<sup>1</sup> Smoking causes endothelial dysfunction<sup>6</sup> and is associated with increased platelet thrombus formation.<sup>5</sup> Small areas of denudation and thrombus deposition are a common finding on the surface of atheromatous plaques<sup>7,8</sup> and are usually subclinical. However, in the presence of an imbalance in the coagulation or fibrinolytic systems, such microthrombi may propagate, ultimately leading to arterial occlusion.

The importance of endogenous tissue plasminogen activator (tPA) release is exemplified by the high rate of spontaneous reperfusion in the infarct-related artery after acute myocardial infarction, occurring in ≈30% of patients within

the first 12 hours.<sup>9-11</sup> It would be anticipated that high plasma tPA concentrations should protect against subsequent coronary events. However, in epidemiological studies of patients with ischemic heart disease<sup>12,13</sup> and in a healthy male population (US Physicians Study),<sup>14</sup> higher total plasma tPA (antigen) concentrations positively predict future coronary events. This is explained by the concomitant elevation of plasminogen activator inhibitor type 1 (PAI-1), which forms a complex with tPA and thereby causes an overall reduction in free tPA "activity."<sup>15,16</sup> It is this free and unbound tPA that is physiologically active and leads to endogenous fibrinolysis. However, the capacity of endothelial cells to release tPA from intracellular storage pools and the rapidity with which this can be mobilized may not necessarily be reflected in the basal circulating plasma concentrations of tPA antigen or activity.<sup>17</sup>

Using the endothelium-dependent vasodilator substance P to stimulate tPA release, we recently described an in vivo model to assess the acute fibrinolytic capacity of the human

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forearm.<sup>18</sup> Moreover, we have been able to demonstrate a reduction in tPA release after inducing experimental "endothelial dysfunction" with nitric oxide synthase inhibition.<sup>19</sup> We therefore hypothesized that cigarette smoking might impair endogenous fibrinolysis by reducing the capacity of the endothelium to release tPA acutely. The aim of the study was to compare substance P-induced tPA release from the forearm vascular bed of smokers and age- and sex-matched nonsmokers.

## Methods

### Subjects

Twelve healthy smokers (5 to 20 cigarettes/d) and 12 age- and sex-matched nonsmokers between 25 and 55 years old participated in the study, which was undertaken with the approval of the local research ethics committee and in accordance with the Declaration of Helsinki. The written informed consent of each subject was obtained before entry into the study.

All subjects were normotensive without a history of diabetes mellitus or vascular disease. Female subjects were premenopausal and not receiving hormonal contraceptives. They were clinically well and taking no regular medications. Control subjects were lifelong nonsmokers and were not exposed to regular environmental tobacco smoke. Smokers had a history of regular daily cigarette smoking of at least 5 years' standing and maintained their normal smoking habits in the week before attendance. None of the subjects received vasoactive or nonsteroidal anti-inflammatory drugs in the week before the study, and all abstained from alcohol for 24 hours before and from food, tobacco, and caffeine-containing drinks on the day of the study. All studies were performed in a quiet, temperature-controlled room maintained at 23.5°C to 24.5°C.

### Intra-Arterial Drug Administration

The brachial artery of the nondominant arm was cannulated with a 27-standard wire gauge steel needle (Cooper's Needle Works Ltd) under local anesthesia. The cannula was attached to a 16-gauge epidural catheter (Portex Ltd), and patency was maintained by infusion of saline (0.9%; Baxter Health Care Ltd) via an IVAC P1000 syringe pump (IVAC Ltd). The total rate of intra-arterial infusions was maintained constant throughout all studies at 1 mL/min. Pharmaceutical-grade substance P (Clinalfa AG) was administered after dissolution in saline.

### Measurements

Blood flow was measured in both forearms by venous occlusion plethysmography as previously described.<sup>18–20</sup> Blood pressure was monitored in the noninfused arm at intervals throughout each study with a semiautomated noninvasive oscillometric sphygmomanometer (Takeda UA 751, Takeda Medical Inc).

Venous cannulas (17-gauge) were inserted into large subcutaneous veins of the antecubital fossae of both arms. Blood (10 mL) was withdrawn simultaneously from each arm and collected into acidified buffered citrate (Biopool Stabilyte, for tPA assays) and citrate (Monovette, for PAI-1 assays) tubes and kept on ice before being centrifuged at 2000g for 30 minutes at 4°C. Platelet-free plasma was decanted and stored at –80°C before assay. Plasma PAI-1 and tPA antigen and activities were determined as previously described<sup>18,19</sup> with an ELISA (Coalza PAI-1 and Coalza tPA, Chromogenix AB) and a photometric method (Coatest PAI-1 and Coaset tPA, Chromogenix AB). Hematocrit was determined by capillary tube centrifugation at baseline and during infusion of 8 pmol/min of substance P. Plasma lipid fractions were measured by an enzymatic colorimetric method (Boehringer Mannheim GmbH Diagnostica). LDL cholesterol was derived according to the method of Friedewald et al.<sup>21</sup>

TABLE 1. Baseline Subject Characteristics

	Nonsmokers	Smokers
Age, y	35±3	34±2
Sex, male:female	10:2	10:2
Body mass index, kg/m <sup>2</sup>	23.9±0.5	24.5±1.1
Mean arterial pressure, mm Hg	92±2	91±2
Heart rate, bpm	59±3	66±2
Fasting plasma glucose, mmol/L	5.3±0.1	5.4±0.1
Total cholesterol, mg/dL	189±14	200±16
LDL cholesterol, mg/dL	113±13	130±16
HDL cholesterol, mg/dL	48±3	37±2*
Triglycerides, mg/dL	138±16	163±27
Baseline hematocrit	0.41±0.01	0.43±0.01

\**P* = 0.01 (unpaired *t* test, smokers vs nonsmokers).

### Study Design

At 9 AM, subjects attended fasted and then rested recumbent throughout each study. Strain gauges and cuffs were applied, and the brachial artery of the nondominant arm was cannulated. Forearm blood flow was measured every 10 minutes. Saline was infused for the first 30 minutes to allow time for equilibration. The final blood flow measurement during saline infusion was taken as the basal forearm blood flow. Thereafter, subjects received intra-arterial substance P at 2, 4, and 8 pmol/min for 10 minutes at each dose.

### Data Analysis and Statistics

This study's population size, on the basis of power calculations derived from previous studies, gives 90% power of detecting an 18% difference in tPA release at a significance level of 5%. Coefficients of repeatability<sup>22</sup> for plasma concentrations of tPA antigen and activity during substance P infusion at 8 pmol/min are 1.6 ng/mL and 1.4 IU/mL, respectively (data on file).

Plethysmographic data were extracted from the Chart data files, and forearm blood flows were calculated for individual venous occlusion cuff inflations by use of a template spreadsheet (Excel version 5.0; Microsoft Corp). Recordings from the first 60 seconds after wrist cuff inflation were not used because of the reflex vasoconstriction this causes.<sup>20</sup> Usually, the last 5 flow recordings in each 3 minute measurement period were calculated and averaged for each arm. Estimated net release of tPA activity and antigen was defined previously<sup>18</sup> as the product of the infused forearm plasma flow (based on the mean hematocrit, Hct, and the infused forearm blood flow, FBF) and the concentration difference between the infused ([tPA]<sub>inf</sub>) and noninfused ([tPA]<sub>noninf</sub>) arms: Estimated net tPA release = FBF × (1 – Hct) × ([tPA]<sub>inf</sub> – [tPA]<sub>noninf</sub>).

Data were examined, where appropriate, by 2-way ANOVA with repeated measures and 2-tailed Student's *t* test using Excel version 5.0 (Microsoft). The area under the curve was calculated for the estimated net release of tPA across the study period. All results are expressed as mean ± SEM. Statistical significance was taken at the 5% level.

## Results

There were no significant differences in baseline characteristics, except that smokers had a slightly lower HDL concentration (Table 1). There were no significant changes in blood pressure, heart rate, hematocrit, or blood flow in the noninfused forearm during the study (data on file; Table 2). In the noninfused arm, plasma tPA antigen concentrations were higher in smokers than nonsmokers (*P* = 0.02; Table 2). There were no significant differences in plasma PAI-1 antigen and activity between the groups.

TABLE 2. Blood Flow and Plasma tPA and PAI-1 Antigen and Activity Concentrations in Both Forearms

	Nonsmokers				Smokers			
	Baseline	Time, min			Baseline	Time, min		
		10	20	30		10	20	30
Substance P dose, pmol/min	0	2	4	8	0	2	4	8
Absolute forearm blood flow, mL · 100 mL <sup>-1</sup> · min <sup>-1</sup>								
Noninfused arm	2.8±0.3	2.9±0.4	2.9±0.4	2.8±0.3	2.8±0.2	2.9±0.3	2.9±0.3	3.0±0.3
Infused arm	3.7±0.4	11.2±1.1	13.5±1.3	16.2±1.5*	3.6±0.3	9.4±0.4	11.5±0.7	14.2±0.8*†
tPA antigen, ng/mL								
Noninfused arm	3.3±0.5	3.4±0.5	3.4±0.5	3.7±0.5	4.0±0.5	4.3±0.5	4.4±0.5	4.4±0.6†
Infused arm	3.2±0.5	4.1±0.6	4.4±0.6	6.2±0.8*	4.1±0.5	4.5±0.6	5.2±0.7	5.9±0.9*
tPA activity, IU/mL								
Noninfused arm	0.8±0.2	0.9±0.2	1.0±0.2	1.3±0.2	0.7±0.1	0.7±0.1	0.8±0.1	1.0±0.2
Infused arm	0.8±0.2	2.1±0.5	2.8±0.5	4.6±0.6*	0.7±0.1	1.1±0.2	1.7±0.4	3.0±0.5*†
PAI-1 antigen, ng/mL								
Noninfused arm	29±7	29±6	28±7	28±6	29±6	26±5	25±5	26±5
Infused arm	28±6	28±7	27±6	28±5	26±5	27±6	26±6	26±5
PAI-1 activity, AU/mL								
Noninfused arm	11.8±1.7	11.8±1.7	12.1±1.6	11.4±1.8	12.0±2.0	11.0±1.7	9.2±1.4	10.2±1.3
Infused arm	10.7±1.6	8.8±1.5	10.8±1.7	9.3±1.5	12.5±1.9	10.6±1.5	10.5±1.2	8.5±1.1

One-way ANOVA: \* $P<0.001$ ; 2-way ANOVA (nonsmokers vs smokers): † $P<0.05$ ; ‡ $P=0.001$ .

Substance P caused dose-dependent increases in forearm blood flow in the infused arm in both smokers and nonsmokers (Table 2, Figure), but the increase in blood flow was greater in nonsmokers ( $P=0.03$ ; 2-way ANOVA, nonsmokers versus smokers). Compared with the noninfused arm (2-way ANOVA), substance P caused dose-dependent increases in plasma concentrations of tPA antigen ( $P<0.001$ ) and activity ( $P<0.001$ ) in the infused arm of both smokers and nonsmokers (Table 2). There were no significant changes in plasma PAI-1 antigen or activity in either group. The increase in plasma tPA activity in the infused arm was greater in the nonsmokers ( $P=0.001$ ; 2-way ANOVA, nonsmokers versus smokers).

Substance P increased the net release of tPA antigen ( $P=0.009$ ) and activity ( $P<0.001$ ) in smokers (Figure). In nonsmokers, substance P increased the net release of tPA antigen ( $P<0.001$ ) and activity ( $P<0.001$ ) significantly more than in smokers ( $P=0.04$  and  $P<0.001$ , respectively; 2-way ANOVA, nonsmokers versus smokers). Compared with the nonsmokers, the area under the curve for net tPA antigen and activity release was reduced by 51% and 53%, respectively, in the smokers.

Subgroup analysis after exclusion of female subjects did not alter the magnitude or the statistical significance of the above findings. Qualitatively, the responses in female smokers and nonsmokers were similar to those observed in the male subjects.

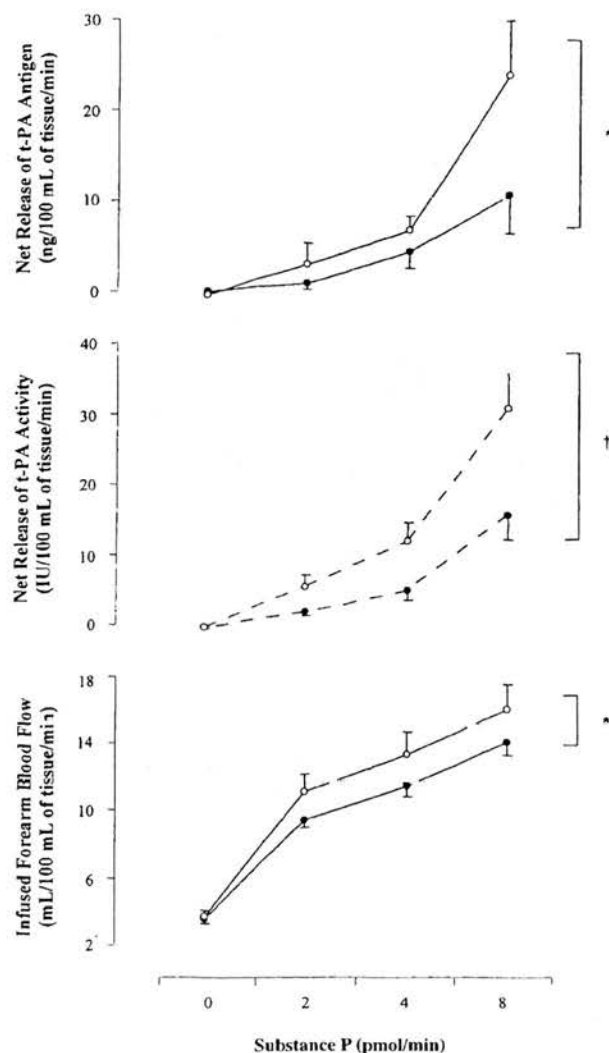
## Discussion

We have shown here, for the first time, that despite higher basal plasma tPA antigen concentrations, cigarette smokers have a markedly impaired capacity of the endothelium to

release tPA acutely. This establishes an important mechanism whereby cigarette smoking can lead to arterial thrombosis and myocardial infarction.

The rapid mobilization of tPA from the endothelium is crucial if endogenous fibrinolysis within the arterial circulation is to be effective, with thrombus dissolution being much more effective if tPA is incorporated during, rather than after, thrombus formation.<sup>23,24</sup> The increased risk of spontaneous thrombosis seen in smokers may therefore plausibly relate to the propagation of thrombus, which would otherwise undergo lysis and remain subclinical. Although cigarette smokers have a higher overall mortality from myocardial infarction than nonsmokers,<sup>25</sup> the in-hospital mortality is lower.<sup>26–28</sup> This apparent paradox can be explained by the observation that the infarct-related artery is more than twice as likely to become patent in current smokers as in nonsmokers after thrombolytic therapy for acute myocardial infarction.<sup>28–30</sup> Indeed, it has been suggested<sup>30</sup> that thrombolytic therapy should only be given to smokers and that alternative strategies such as primary angioplasty should be used in nonsmokers. These observations are consistent with the present findings because it might be anticipated that patients with impaired endothelial cell tPA release would benefit most from thrombolytic therapy, whereas those with a normal endogenous fibrinolytic capacity are more likely to have tPA-resistant thrombus, which would respond less favorably.

Our findings in smokers are consistent with the previous observational data<sup>12–14</sup> that increased basal plasma concentrations of tPA antigen are associated with future coronary events. The assessment of endogenous fibrinolysis has previously relied on measurement of basal plasma tPA concentrations and the acute release of tPA in response to venous



Infused forearm blood flow and the net release of tPA antigen and activity in smokers (●) and nonsmokers (○).  $P < 0.001$  (1-way ANOVA) for all responses. Two-way ANOVA (nonsmokers vs smokers): \* $P < 0.05$ ; † $P < 0.001$ .

occlusion, systemic desmopressin infusion, or exercise.<sup>31–34</sup> However, because of confounding systemic effects and the nonuniformity of the stimuli applied, these responses can be variable and give only a relatively crude measure of fibrinolytic capacity. Moreover, although it has previously been shown that systemic desmopressin infusion causes less tPA release in smokers,<sup>31</sup> this effect may not be directly endothelium-dependent.<sup>35</sup> In contrast, we have used locally active doses of substance P to provide a more precise pharmacological stimulus to the endothelium and to cause a substantial and dose-dependent local release of tPA.<sup>18,19</sup> This has allowed us to demonstrate a distinct and marked inhibition of stimulated endothelial tPA release in smokers.

Although thrombin is more physiologically relevant to acute tPA release than substance P, we have used the latter because its vascular actions are endothelium-dependent,<sup>36</sup> mediated in part through nitric oxide,<sup>37</sup> and its administration intra-arterially is safe and well tolerated.<sup>38</sup> Consistent with previous workers,<sup>6,39,40</sup> we have also found an attenuation of

the endothelium-dependent forearm blood flow responses in smokers. This inhibition of both the blood flow and tPA response may, in part, relate to an impairment of the L-arginine:nitric oxide pathway in smokers.<sup>19,39</sup> Although differences exist,<sup>41</sup> the forearm model may provide a useful surrogate for the coronary vascular bed<sup>42,43</sup> and permits a readily accessible and reliable assessment of endothelial cell function. However, the present findings need to be confirmed in the coronary circulation.

We have studied the sustained effect of chronic smoking in a selected healthy and predominantly male population at a single time point. Although total and LDL cholesterol concentrations were similar in smokers and nonsmokers, HDL cholesterol concentrations were slightly lower in smokers. This is not unexpected, because cigarette smoking is known to be associated with a selective reduction in HDL cholesterol concentrations.<sup>44,45</sup> However, the application of this model to other conditions associated with endothelial dysfunction, such as dyslipidemia, is warranted. Finally, because hormonal status influences fibrinolytic parameters,<sup>46</sup> the assessment of the acute fibrinolytic capacity in premenopausal and postmenopausal women and the modulating effect of hormonal therapy will also be of particular interest.

In conclusion, we have demonstrated a major impairment of tPA release from the vascular endothelium of smokers. Our findings suggest that the fundamental mechanism whereby cigarette smoking causes arterial thrombosis and myocardial infarction relates, at least in part, to impairment of the acute endogenous fibrinolytic capacity.

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## **PUBLICATION 12**

# Effects of acute methionine loading and vitamin C on endogenous fibrinolysis, endothelium-dependent vasomotion and platelet aggregation

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## ABSTRACT

We assessed forearm blood flow and plasma fibrinolytic factors in eight healthy males who received unilateral brachial artery infusions of the endothelium-dependent vasodilator, substance P, and the endothelium-independent vasodilator, sodium nitroprusside. These measurements, together with platelet aggregation studies, were performed on four occasions after double-blind randomized ingestion of placebo, methionine (0.1 mg/kg), vitamin C (2 g) and methionine plus vitamin C. Blood flow and platelet aggregation responses were unaffected by methionine loading. Substance P caused dose-dependent increases in plasma tissue plasminogen activator (t-PA) antigen (from  $3.0 \pm 0.1$  to  $4.7 \pm 0.4$  ng/ml;  $P < 0.001$ ) and activity (from  $1.2 \pm 0.2$  to  $4.2 \pm 0.4$  i.u./ml;  $P < 0.001$ ), which were augmented during acute methionine loading ( $4.7 \pm 0.4$  to  $5.6 \pm 0.5$  ng/ml and  $4.2 \pm 0.4$  to  $5.5 \pm 0.9$  i.u./ml respectively;  $P \leq 0.05$ ). Moreover, the estimated net release of t-PA was enhanced during methionine loading (two-way ANOVA;  $P = 0.02$ ), but this was unaffected by vitamin C supplementation. We conclude that, in the absence of alterations in endothelium-dependent vasomotion or platelet aggregation, substance P-induced t-PA release is enhanced following methionine loading. This suggests that the acute endogenous fibrinolytic capacity is augmented during acute hyperhomocysteinaemia in healthy humans via an oxidation-independent mechanism.

## INTRODUCTION

Elevated plasma homocysteine concentrations are an independent risk factor for myocardial infarction [1], are associated with thrombophilia, and confer a more than 20-fold increased risk of coronary artery disease [2]. Severe hyperhomocysteinaemia is rare, but moderate hyperhomocysteinaemia is more common [3–5] and is present in ~30% of patients with premature coronary artery disease [2]. In contrast with serum lipid fractions, homocysteine concentrations are correlated only weakly with the extent of coronary atheroma [6], suggesting that

the association with coronary events is more likely to represent thrombogenicity or plaque rupture rather than atherogenicity.

It has been recognized for over 20 years that elevated plasma concentrations of homocysteine cause endothelial cell injury and denudation in animal models [7,8]. However, more recent studies have confirmed that endothelial dysfunction is also present in human subjects with hyperhomocysteinaemia [9–12], and that this may be reversed by lowering plasma homocysteine concentrations using pyridoxine and folate therapy [13]. The mechanism whereby hyperhomocysteinaemia produces

**Key words:** blood flow, endothelium, hyperhomocysteinaemia, plasminogen activators, platelet aggregation.

**Abbreviations:** t-PA, tissue plasminogen activator; PAI, plasminogen activator inhibitor; a.u., arbitrary units.

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endothelial dysfunction may, in part, relate to oxidative stress and superoxide generation [14]. Indeed, acute antioxidant administration using vitamin C appears to reverse the impairment of flow-associated vasodilatation induced by methionine loading in healthy volunteers [15].

The fibrinolytic factor tissue plasminogen activator (t-PA) and its inhibitor, plasminogen activator inhibitor type 1 (PAI-1), are potentially important endothelium-derived mediators that are intimately linked to the risk of thrombosis. The time course of t-PA release is important, since clot dissolution is much more effective if t-PA is incorporated during clot formation rather than following completion [16,17]. However, the capacity of endothelial cells to release t-PA from intracellular storage pools, and the rapidity with which it can be mobilized, may not necessarily be reflected in the basal circulating plasma concentrations of t-PA antigen or its activity [18,19]. We have shown, using bilateral forearm venous occlusion plethysmography and unilateral brachial artery infusions, that the forearm release of t-PA can be determined *in vivo* in humans [20]. This model permits a more precise pharmacological stimulus to be applied to the endothelium in a well defined and reproducible manner. Moreover, on applying this model to healthy subjects with a smoking habit, we have shown that, despite higher basal plasma antigen concentrations, cigarette smokers have a markedly impaired capacity to release t-PA acutely [18].

In a longitudinal study of stroke patients, plasma t-PA concentrations were found to be an independent discriminator, and were correlated directly with plasma homocysteine concentrations [21]. Moreover, methionine loading causes more pronounced alterations in basal fibrinolytic parameters of patients with premature vascular disease [22]. Although it would appear that hyperhomocysteinaemia may be associated with alterations in endogenous fibrinolysis, it is not known whether changes in plasma homocysteine concentrations can influence the acute release of t-PA from the endothelium.

In addition to procoagulant effects on the vessel wall, hyperhomocysteinaemia may induce vascular occlusion through platelet actions [23,24]. Animal studies [23,25] indicate that hyperhomocysteinaemia enhances platelet aggregation. However, the effects of acute elevations of plasma homocysteine concentrations on the activity of platelets in humans are currently unknown.

We hypothesized that experimental hyperhomocysteinaemia, produced by methionine loading, would impair substance P-induced forearm vasodilatation and t-PA release, and enhance platelet aggregation. Moreover, given the potential role of oxidative stress, we further hypothesized that vitamin C supplementation would reverse these potential derangements in endothelial and platelet function. Therefore the aims of the present study were to examine the effects of increased plasma homo-

cysteine concentrations, following an oral methionine load, on acute endogenous t-PA release, endothelium-dependent vasomotion and platelet aggregation, and to determine the influence of vitamin C supplementation on these responses.

## METHODS

### Subjects

Eight healthy male non-smokers, aged between 20 and 42 (mean 30) years, participated in a four-phase study, which was undertaken with the approval of the local research ethics committee and in accordance with the Declaration of Helsinki (1989) of the World Medical Association. The written informed consent of each subject was obtained before entry into the study. Volunteers were normotensive, with normal serum cholesterol, folate and vitamin B<sub>12</sub> levels. None of the subjects received vasoactive or non-steroidal anti-inflammatory drugs in the week before each phase of the study, and all abstained from alcohol for 24 h, and from caffeine-containing foods for at least 12 h, before each study. All studies were carried out in a quiet, temperature-controlled room maintained at 22–24 °C.

### Drugs

Pharmaceutical-grade substance P (Clinalfa AG, Läufelfingen, Switzerland) and sodium nitroprusside (Nipride; Roche, Welwyn Garden City, U.K.) were administered following dissolution in 0.9% saline (Baxter Healthcare Ltd, Thetford, Norfolk, U.K.). In order to maintain blinding, methionine (0.1 mg/kg; Evans Medical Ltd, Leatherhead, Surrey, U.K.) [12], vitamin C (2 g; Redoxen; Roche) and placebo were administered following dissolution in an orange-flavoured drink containing no vitamin C.

### Intra-arterial administration

The brachial artery of the non-dominant arm was cannulated with a 27-standard wire gauge steel needle (Cooper's Needle Works Ltd, Birmingham, U.K.) under 1% lignocaine (Xylocaine; Astra Pharmaceutical Ltd, Kings Langley, Herts., U.K.) local anaesthesia, and attached to a 16 gauge epidural catheter (Portex Ltd, Hythe, Kent, U.K.). Patency was maintained by infusion of saline via an IVAC P1000 syringe pump (IVAC Ltd, Basingstoke, Hants., U.K.). The total rate of intra-arterial infusion was maintained constant throughout all studies at 1 ml/min.

### Forearm blood flow and haemodynamics

Blood flow was measured in both forearms by venous occlusion plethysmography using mercury-in-silastic

strain gauges applied to the widest part of the forearm [26]. During measurement periods, the hands were excluded from the circulation by rapid inflation of wrist cuffs to a pressure of 220 mmHg using E20 Rapid Cuff Inflators (D. E. Hokanson Inc., Washington, DC, U.S.A.). Upper-arm cuffs were inflated intermittently to 40 mmHg for 10 s in every 15 s to achieve venous occlusion and obtain plethysmographic recordings. Analogue voltage output from an EC-4 Strain Gauge Plethysmograph (D. E. Hokanson Inc.) was processed by a MacLab<sup>®</sup> analogue-to-digital converter and Chart<sup>®</sup> v3.3.8 software (AD Instruments Ltd, Castle Hill, NSW, Australia) and recorded on to a MacIntosh Classic II computer (Apple Computers Inc.). Calibration was achieved using the internal standard of the plethysmograph.

Blood pressure and heart rate were monitored in the non-infused arm at intervals throughout each study using a semi-automated non-invasive oscillometric sphygmomanometer (Takeda UA 751; Takeda Medical Inc., Tokyo, Japan) [27].

## Assays

### t-PA and PAI-1

Venous cannulae (17G) were inserted into large subcutaneous veins of the antecubital fossa in both arms. A 10 ml blood sample was withdrawn simultaneously from each arm and collected into acidified buffered citrate (Biopool<sup>®</sup>; Stabilyte, Umeå, Sweden; for t-PA assays) and citrate (Monovette<sup>®</sup>; Sarstedt, Nümbrecht, Germany; for PAI-1 assays) tubes, and kept on ice before being centrifuged at 2000 g for 30 min at 4 °C. Platelet-free plasma was decanted and stored at -80 °C before assay [28].

Plasma PAI and t-PA antigen concentrations were determined using ELISAs: Coaliza<sup>®</sup> PAI-1 [29] and Coaliza<sup>®</sup> t-PA [30] (Chromogenix AB, Mölndal, Sweden) respectively. Plasma PAI and t-PA activities were determined by a photometric method: Coatest<sup>®</sup> PAI-1 [31] and Coatest<sup>®</sup> t-PA [32] (Chromogenix AB). Intra-assay coefficients of variation were 7.0% and 5.5% respectively for t-PA and PAI-1 antigen, and 4.0% and 2.4% respectively for activity. Inter-assay coefficients of variation were 4.0%, 7.3%, 4.0% and 7.6% respectively. The sensitivities of the assays were 0.5 ng/ml, 2.5 ng/ml, 0.10 i.u./ml and 5 arbitrary units (a.u.)/ml respectively. The haematocrit was determined from the infused forearm at baseline and during infusion of 8 pmol of substance P/min.

### Homocysteine and vitamin C

Plasma total homocysteine concentrations were measured, as described previously [33], in blood collected in lithium/heparin (Monovette<sup>®</sup>). In brief, following

addition of 2-mercaptoethylamine and reduction with tri-*n*-butylphosphine, plasma was deproteinized using 10% trichloroacetic acid and derivatized with 7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonic acid at 60 °C for 1 h. Derivatized thiols were separated by HPLC (C<sub>18</sub> columns; Millipore, Milford, MA, U.S.A.; mobile phase 6% acetonitrile in 0.1 mol/l KH<sub>2</sub>PO<sub>4</sub> adjusted to pH 2.15 with H<sub>3</sub>PO<sub>4</sub>; flow rate 0.8 ml/min) and detected in a fluorescence detector (Perkin Elmer LS30; excitation 385 nm, emission 515 nm). Chromatograms were analysed using computer software (JCL 6000 Chromatography Data System; Jones Chromatography, Lakewood, CO, U.S.A.) and expressed as the quotient of the homocysteine peak area (retention time 5.3 min) and the internal standard peak area (retention time 3.3 min). The peak ratio showed a linear relationship with homocysteine concentration in spiked plasma samples (< 2.5 to > 40 µmol/l;  $r^2 = 0.997$ ). Inter- and intra-assay coefficients of variation were < 12% and < 8% respectively.

Blood samples in lithium/heparin (Monovette<sup>®</sup>) were centrifuged immediately, and 500 µl of plasma was separated before being rapidly snap-frozen following addition of 500 µl of 5% metaphosphoric acid. Plasma vitamin C concentrations were determined using an enzymic colorimetric method, as previously described [34] (Cobas Bio centrifugal analyser equipped with a fluorescence attachment).

### Platelet aggregometry

A 30 ml sample of blood was collected into trisodium citrate tubes (Monovette<sup>®</sup>) and immediately centrifuged at 120 g for 10 min to obtain platelet-rich plasma, which was aspirated and pre-warmed to 37 °C. Aggregation studies were performed, 30–40 min after blood sampling, using a standard optical technique (Chronolog Ca560 aggregometer; Labmedics, Stockport, U.K.) as described previously [35]. The maximum aggregation attained within 7 min of the addition of each ADP concentration (1–8 µmol/l) was recorded, and expressed as a percentage of the response to 8 µmol/l ADP. Measurement of platelet counts and haematocrit were performed using an automated Coulter counter (ACT.8 Coulter Counter; Beckman-Coulter, High Wycombe, U.K.).

## Study design

All subjects attended at 08.00 hours on each of four study days at least 1 week apart, and participated in each of the four study phases: double placebo, methionine plus placebo, vitamin C plus placebo, and methionine plus vitamin C. Blood samples were obtained for the measurement of plasma homocysteine concentrations and platelet aggregation before and 6 h after the ingestion of double placebo, methionine plus placebo, vitamin C plus placebo or methionine plus vitamin C, administered in a double-

**Table 1** Absolute blood flow in the infused and non-infused forearms during substance P infusion: endothelium-dependent vasomotion

SP, substance P. The significance of substance P dose-response relationships in the infused arm was assessed by one-way ANOVA. Differences between placebo and methionine were not significant by two-way ANOVA.

		Blood flow (ml · min <sup>-1</sup> · 100 ml <sup>-1</sup> )								
		Infused arm					Non-infused arm			
Treatment	[SP] (pmol/min) ...	0	2	4	8	<i>P</i>	0	2	4	8
Placebo supplementation										
Placebo		5.8 ± 1.0	13.9 ± 0.9	17.4 ± 1.2	22.1 ± 1.9	< 0.001	3.5 ± 0.3	4.3 ± 0.5	4.1 ± 0.4	4.4 ± 0.5
Methionine		4.9 ± 0.5	12.8 ± 1.4	14.9 ± 1.3	17.0 ± 1.8	< 0.001	3.2 ± 0.3	3.2 ± 0.2	2.9 ± 0.3	3.7 ± 0.4
Vitamin C supplementation										
Placebo		4.6 ± 0.8	12.2 ± 2.1	14.7 ± 2.2	18.2 ± 2.8	< 0.001	3.2 ± 0.5	3.4 ± 0.5	3.5 ± 0.4	3.6 ± 0.4
Methionine		4.7 ± 0.9	12.3 ± 1.5	15.6 ± 2.3	20.3 ± 3.2	< 0.001	3.2 ± 0.4	3.6 ± 0.5	3.6 ± 0.5	3.9 ± 0.6

blind randomized manner. The studies were timed to coincide with the peak plasma homocysteine concentration, which occurs between 4 and 8 h [12,36].

At 13.30 hours on each study day, subjects rested recumbent; strain gauges and cuffs were applied and the brachial artery of the non-dominant arm was cannulated. Measurements of forearm blood flow were made every 10 min, between 3 and 6 min into each infusion period. Before substance P and sodium nitroprusside administration, saline was infused for 30 min to allow time for equilibration, and the final blood flow measurement during saline infusion was taken as basal forearm blood flow. Substance P was infused at 2, 4 and 8 pmol/min for 10 min at each dose [18,20], and sodium nitroprusside was infused at 2, 4 and 8 µg/min for 10 min at each dose [20]. The order of substance P and sodium nitroprusside administration was randomized, although for each subject the order was maintained on each of the four occasions. Plasma fibrinolytic parameters were assessed at baseline and at the end of each dose of substance P, but not during sodium nitroprusside infusion, since the latter does not affect plasma t-PA or PAI-1 concentrations in this model [20].

### Data analysis and statistics

The study population size, based on power calculations derived from previous studies [20], gives 90% power of detecting a 21% difference in t-PA release at a significance level of 5%. Coefficients of repeatability [37] for plasma concentrations of t-PA antigen and activity during substance P infusion at 8 pmol/min are 1.6 ng/ml and 1.4 i.u./ml respectively (results not shown).

Plethysmographic and aggregometry data were extracted from the Chart<sup>®</sup> data files. Forearm blood flows were calculated for individual venous occlusion cuff inflations by use of a template spreadsheet (Excel v5.0; Microsoft Corp.). Recordings from the first 60 s

after wrist cuff inflation were not used, because of the variability in blood flow this causes. Usually, the last five flow recordings in each 3 min measurement period were calculated and averaged for each arm. Estimated net release of t-PA activity and antigen was defined previously [20] as the product of the infused forearm plasma flow [based on the mean haematocrit (Hct) and the infused forearm blood flow (FBF)] and the concentration difference between the infused ([t-PA]<sub>inf</sub>) and non-infused ([t-PA]<sub>non-inf</sub>) arms:

$$\text{Estimated net t-PA release} = \text{FBF} \times (1 - \text{Hct}) \times ([\text{t-PA}]_{\text{inf}} - [\text{t-PA}]_{\text{non-inf}})$$

Data were examined, where appropriate, by two-way ANOVA for repeated measures and two-tailed paired Student's *t*-test using Excel v5.0 (Microsoft). All results are expressed as means ± S.E.M. Statistical significance was taken at the 5% level.

## RESULTS

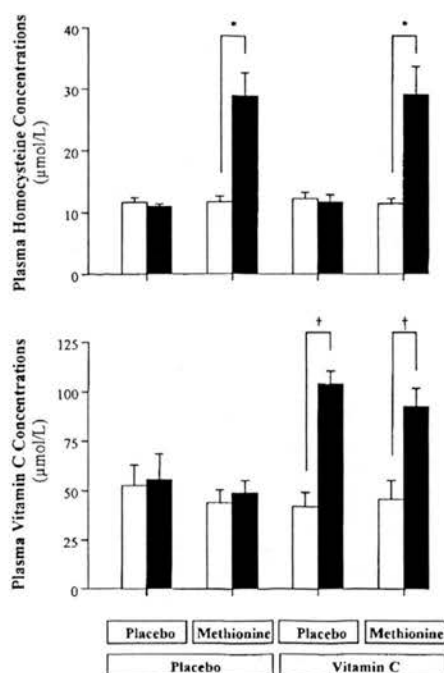
There were no significant differences in baseline blood pressure, heart rate, haematocrit (results not shown) or forearm blood flow between the four study days (Tables 1 and 2). On each of the study days, blood pressure, heart rate, haematocrit (results not shown) and non-infused forearm blood flow (Tables 1 and 2) did not change during the study. However, plasma homocysteine concentrations (Figure 1) were elevated in all subjects following methionine plus placebo or methionine plus vitamin C (*P* = 0.01; paired *t*-test), but not after double placebo or placebo plus vitamin C. Plasma vitamin C concentrations increased after vitamin C plus placebo or vitamin C plus methionine (*P* < 0.001), but not after double placebo or placebo plus methionine (Figure 1).



**Table 2** Absolute blood flow in the infused and non-infused forearms during sodium nitroprusside infusion: endothelium-independent vasomotion

SNP, sodium nitroprusside. The significance of sodium nitroprusside dose-response relationships in the infused arm was assessed by one-way ANOVA. Differences between placebo and methionine were not significant by two-way ANOVA.

		Blood flow (ml · min <sup>-1</sup> · 100 ml <sup>-1</sup> )								
		Infused arm					Non-infused arm			
Treatment	SNP (μg/min) ...	0	2	4	8	<i>P</i>	0	2	4	8
Placebo supplementation										
Placebo		4.2 ± 0.3	16.9 ± 3.9	20.2 ± 4.1	20.7 ± 3.1	< 0.001	3.4 ± 0.2	3.7 ± 0.3	3.9 ± 0.4	3.6 ± 0.3
Methionine		4.1 ± 0.4	10.8 ± 0.7	14.0 ± 1.1	16.9 ± 1.4	< 0.001	3.1 ± 0.2	3.0 ± 0.2	3.5 ± 0.3	3.1 ± 0.3
Vitamin C supplementation										
Placebo		3.6 ± 0.4	11.5 ± 2.3	15.9 ± 2.9	20.6 ± 4.4	< 0.001	2.6 ± 0.2	2.7 ± 0.2	2.8 ± 0.2	3.1 ± 0.5
Methionine		4.1 ± 0.4	13.2 ± 3.3	17.3 ± 3.9	20.8 ± 3.4	< 0.001	3.2 ± 0.3	3.3 ± 0.4	3.5 ± 0.4	3.5 ± 0.3



**Figure 1** Plasma homocysteine (upper panel) and vitamin C (lower panel) concentrations at baseline (□) and 6 h post-ingestion (■) of double placebo, methionine plus placebo, vitamin C plus placebo or methionine plus vitamin C. Significance of differences (paired *t*-test): \* $P \leq 0.01$ , † $P < 0.001$ .

### Endothelium-dependent and -independent vasomotion

Sodium nitroprusside and substance P caused dose-dependent increases in blood flow of the infused forearm (ANOVA:  $P < 0.001$  for all; Tables 1 and 2) on all four study days. The blood flow responses to both sodium nitroprusside and substance P were similar on each of the

study days, with no significant differences in the magnitude of the responses. There was no time order effect of the blood flow responses to substance P or sodium nitroprusside.

### Endogenous fibrinolysis

There were no significant differences between baseline plasma t-PA and PAI-1 concentrations on each of the four study days. Substance P caused dose-dependent increases in plasma t-PA antigen concentration and activity in the infused, but not the non-infused, forearm on each of the four study days (Table 3). Moreover, the concentration difference between the infused and non-infused forearms also increased significantly with substance P infusion (Table 3). Estimated net release of t-PA antigen and activity demonstrated dose-dependent increases (ANOVA:  $P < 0.001$  for all; Figure 2), which were unaffected by co-administration of vitamin C. Substance P-induced increases in t-PA plasma concentrations and forearm release were significantly greater following methionine loading than after placebo (Table 3 and Figure 2).

At 6 h after administration of double placebo, methionine plus placebo, vitamin C plus placebo and methionine plus vitamin C, basal plasma PAI-1 antigen concentrations were  $20 \pm 5$ ,  $18 \pm 3$ ,  $20 \pm 2$  and  $16 \pm 2$  ng/ml respectively, and PAI-1 activities were  $8 \pm 1$ ,  $7 \pm 2$ ,  $8 \pm 1$  and  $7 \pm 1$  a.u./ml respectively. There were no significant differences between the study days.

### Platelet aggregation

There were no differences in platelet counts before or 6 h after the administration of double placebo [ $(442 \pm 27) \times 10^9/\text{l}$  and  $(415 \pm 17) \times 10^9/\text{l}$  respectively] or methionine plus placebo [ $(429 \pm 32) \times 10^9/\text{l}$  and  $(411 \pm 25) \times 10^9/\text{l}$  respectively]. There were no significant changes in the concentration-response curves for ADP-induced platelet aggregation in platelet-rich plasma 6 h

**Table 3** Plasma t-PA antigen concentration and activity in the infused and non-infused forearms during substance P infusion in the presence and absence of vitamin C supplementation

SP, substance P; NS, not significant. Estimated net release is expressed as ng or i.u. per min per 100 ml of tissue. *P* values are given for the substance P dose-response relationship (one-way ANOVA). *P* values are also given for comparisons of placebo with methionine (two-way ANOVA)

	Placebo					Methionine						
SP infusion (pmol/min) ...	0	2	4	8	<i>P</i>	0	2	4	8	<i>P</i>	<i>P</i> (2-way)	
Placebo supplementation												
t-PA antigen (ng/ml)												
Infused arm	3.0 ± 0.1	3.5 ± 0.2	3.9 ± 0.2	4.7 ± 0.4	≤ 0.001	3.3 ± 0.2	4.1 ± 0.5	4.5 ± 0.2	5.6 ± 0.5	≤ 0.01	≤ 0.01	
Non-infused arm	3.1 ± 0.1	3.4 ± 0.2	3.4 ± 0.2	3.5 ± 0.2	NS	3.3 ± 0.2	3.2 ± 0.1	3.3 ± 0.2	3.7 ± 0.2	NS		
Forearm difference	−0.1 ± 0.1	0.1 ± 0.2	0.5 ± 0.2	1.2 ± 0.3	≤ 0.001	0.0 ± 0.2	0.8 ± 0.4	1.2 ± 0.2	1.9 ± 0.5	≤ 0.01	≤ 0.01	
Net release (ng · min <sup>−1</sup> · 100 ml <sup>−1</sup> )	−0.4 ± 0.2	0.8 ± 1.9	5.8 ± 2.0	15.8 ± 4.0	≤ 0.001	0.1 ± 0.6	5.8 ± 2.5	11.4 ± 3.0	20.4 ± 5.7	≤ 0.01		
t-PA activity (i.u./ml)												
Infused arm	1.2 ± 0.2	2.1 ± 0.4	2.6 ± 0.4	4.2 ± 0.4	≤ 0.001	1.5 ± 0.3	2.4 ± 0.4	3.5 ± 0.6	5.5 ± 0.9	≤ 0.001	≤ 0.05	
Non-infused arm	1.5 ± 0.3	1.7 ± 0.5	1.5 ± 0.2	1.9 ± 0.2	NS	1.5 ± 0.3	1.6 ± 0.2	1.9 ± 0.4	2.3 ± 0.4	NS		
Forearm difference	−0.3 ± 0.1	0.4 ± 0.2	1.1 ± 0.2	2.3 ± 0.4	≤ 0.001	−0.1 ± 0.2	0.8 ± 0.3	1.6 ± 0.4	3.2 ± 0.7	≤ 0.001	≤ 0.05	
Net release (i.u. · min <sup>−1</sup> · 100 ml <sup>−1</sup> )	−1.0 ± 0.7	3.3 ± 1.9	11.6 ± 2.4	31.6 ± 7.1	≤ 0.001	−0.4 ± 0.4	6.8 ± 2.6	14.4 ± 4.0	32.2 ± 7.6	≤ 0.001		
Vitamin C supplementation												
t-PA antigen (ng/ml)												
Infused arm	3.1 ± 0.2	3.6 ± 0.2	3.9 ± 0.3	4.3 ± 0.4	≤ 0.05	3.1 ± 0.3	3.5 ± 0.4	4.1 ± 0.5	5.1 ± 0.7	≤ 0.05		
Non-infused arm	3.4 ± 0.2	3.3 ± 0.2	3.4 ± 0.2	3.6 ± 0.3	NS	3.2 ± 0.4	3.1 ± 0.4	3.4 ± 0.4	3.5 ± 0.3	NS		
Forearm difference	−0.3 ± 0.1	0.3 ± 0.1	0.5 ± 0.3	0.7 ± 0.3	≤ 0.05	−0.1 ± 0.1	0.4 ± 0.1	0.7 ± 0.2	1.6 ± 0.5	≤ 0.001	≤ 0.05	
Net release (ng · min <sup>−1</sup> · 100 ml <sup>−1</sup> )	−0.8 ± 0.2	2.2 ± 1.0	5.1 ± 2.8	8.9 ± 4.0	≤ 0.05	−0.2 ± 0.2	3.3 ± 1.1	5.0 ± 1.4	17.7 ± 5.4	≤ 0.001		
t-PA activity (i.u./ml)												
Infused arm	1.3 ± 0.3	2.2 ± 0.3	2.9 ± 0.5	4.0 ± 0.6	≤ 0.001	1.5 ± 0.2	2.8 ± 0.4	3.7 ± 0.9	5.1 ± 0.9	≤ 0.01	≤ 0.11	
Non-infused arm	1.5 ± 0.3	1.5 ± 0.3	1.4 ± 0.3	1.8 ± 0.3	NS	1.3 ± 0.2	1.6 ± 0.3	1.6 ± 0.3	2.0 ± 0.4	NS		
Forearm difference	−0.2 ± 0.1	0.7 ± 0.2	1.5 ± 0.5	2.3 ± 0.6	≤ 0.001	0.2 ± 0.1	1.2 ± 0.3	2.1 ± 0.8	3.1 ± 0.8	≤ 0.01	≤ 0.11	
Net release (i.u. · min <sup>−1</sup> · 100 ml <sup>−1</sup> )	−0.5 ± 0.2	5.6 ± 2.4	15.7 ± 5.8	29.7 ± 9.1	≤ 0.01	0.4 ± 0.4	7.6 ± 1.8	14.9 ± 5.0	34.7 ± 10.0	≤ 0.01		

after oral administration of double placebo or methionine plus placebo (Figure 3). Maximal aggregation (theoretical maximum 80 mV) induced by 8  $\mu$ M ADP was  $68 \pm 4$  and  $65 \pm 3$  mV following placebo and methionine administration respectively.

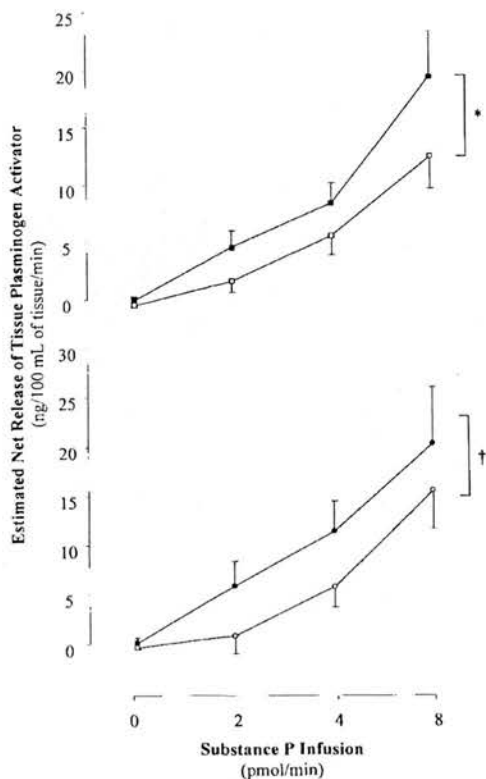
## DISCUSSION

In the absence of alterations in endothelium-dependent vasomotion and platelet aggregation, we report, for the first time, a modest enhancement of substance P-induced t-PA release following methionine loading. This effect does not appear to be mediated through oxidative stress, since co-administration of vitamin C had no effect on this response. Thus it would appear that the acute endogenous fibrinolytic capacity is augmented during acute hyperhomocysteinaemia in healthy humans.

The importance of endogenous t-PA release is exemplified by the high rate of spontaneous reperfusion in the

infarct-related artery after acute myocardial infarction; this occurs in around 30% of patients within the first 12 h [38–40]. It would be anticipated that high plasma t-PA concentrations should protect against subsequent coronary events. However, in epidemiological studies of patients with ischaemic heart disease [41,42], and in prospective studies in healthy populations [43,44], higher total plasma t-PA (antigen) concentrations positively and independently predict future coronary events. This may, in part, be explained by the concomitant elevation of PAI-1, which forms a complex with t-PA and thereby causes an overall decrease in free t-PA 'activity' [45,46]. It is the free and unbound t-PA which is physiologically active and leads to endogenous fibrinolysis. Moreover, in the Northwick Park Heart Study [46] small changes (25%) in fibrinolytic activity were associated with significant increases in the risk of sustaining a myocardial infarction or sudden cardiac death.

The findings of the present study suggest that acute hyperhomocysteinaemia potentiates stimulated t-PA



**Figure 2** Estimated net release of t-PA during incremental substance P infusion

Upper panel: □, placebo plus placebo or vitamin C ( $n = 16$ ); ■, methionine plus placebo or vitamin C ( $n = 16$ ). Lower panel: ○, double placebo ( $n = 8$ ); ●, methionine plus placebo ( $n = 8$ ). Significance of differences: one-way ANOVA,  $P < 0.001$  for all (substance P dose-response); two-way ANOVA,  $*P = 0.02$ ,  $†P = 0.06$  (placebo compared with methionine)

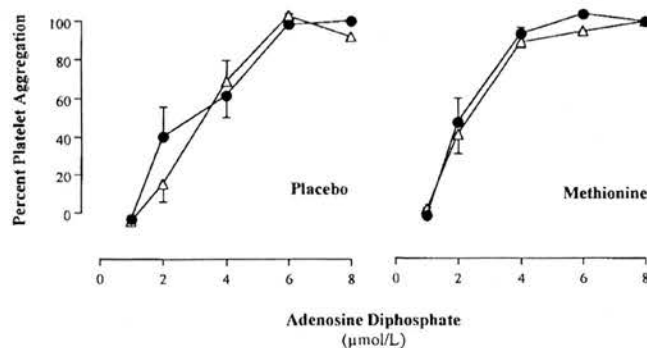
release. This may be a consequence of acute endothelial cell injury or perturbation, which results in an enhanced response on further provocation. However, chronic

hyperhomocysteinaemia may have very different effects, which may include chronic endothelial injury, desensitization and a reduced t-PA response. The results of the present study cannot be extrapolated to chronic hyperhomocysteinaemia, and this requires further careful investigation.

In general, platelet aggregation is sensitive to defects in platelet function rather than to increased platelet activity. However, previous animal studies have indicated that acute methionine loading can enhance platelet aggregation in response to ADP and thrombin [25]. In the light of these findings, we felt that assessment of platelet aggregation could be a useful indicator in our clinical study. However, we have found no evidence of an effect of methionine loading on platelet aggregation in response to ADP. This suggests that hyperhomocysteinaemia is unlikely to alter platelet activity in humans.

In contrast with previous workers [12,15], we have not found an impairment of endothelium-dependent vasomotion in acute hyperhomocysteinaemia. This is despite the use of a comparable regimen of oral methionine loading and a large rise in plasma homocysteine concentrations. However, previous studies [12,15] have used flow-associated dilatation as a non-invasive method of assessing conduit artery endothelial function. In the present study, we have predominantly assessed the function of endothelium within the resistance vessel bed, in terms of the capacity both to release t-PA and to increase forearm blood flow. Conduit artery and microvascular endothelial cells have distinct phenotypic differences, and this may contribute to the apparent disparity in the responses to the same acute insult. Moreover, responses to mechanical rather than pharmacological stimuli may also differ.

Recently, two studies [47,48] have assessed endothelium-dependent resistance vessel function following acute methionine loading. Using the endothelium-dependent vasodilator acetylcholine, these studies have demonstrated either an impairment of [47], or no effect on [48], forearm resistance vessel endothelial



**Figure 3** Platelet aggregation in response to incremental doses of ADP at baseline (●) and 6 h post-ingestion of double placebo or methionine plus placebo (△)

function. These apparently contradictory findings may, in part, reflect the differing study designs. Using a study design similar to that of Kanani and colleagues [47], we achieved similar increases in plasma homocysteine concentrations with acute methionine loading, but failed to detect a significant difference in endothelium-dependent vasodilatation using substance P. This may be explained by the differing signal transduction pathways involved in acetylcholine- and substance P-induced vasodilatation [49,50]. Indeed, previous studies assessing endothelial function, using both substance P and acetylcholine administration, have documented either concordant [51,52] or discordant [53,54] responses that are, in part, dependent upon the disease processes under investigation. Moreover, we chose to use substance P as an endothelial cell stimulant because acetylcholine does not induce the acute release of t-PA in the human forearm [55]. These observations highlight the important differences between the various agents used to stimulate the endothelium, as well as the differing manifestations of endothelial dysfunction that are dependent on the nature of the underlying cellular injury.

In summary, we have found a modest and selective enhancement of substance P-induced t-PA release following methionine loading. It would appear that the acute endogenous fibrinolytic capacity is augmented during acute hyperhomocysteinaemia in healthy humans via an oxidation-independent mechanism.

## ACKNOWLEDGMENTS

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**PUBLICATION 13**

## Short-term effects of transdermal nicotine on acute tissue plasminogen activator release in vivo in man

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### Abstract

**Objective:** Cigarette smoking impairs peripheral endothelium-dependent vasodilatation and acute tissue plasminogen activator (t-PA) release in man. The aim of the study was to determine if this endothelial dysfunction is, in part, mediated by the effects of nicotine. **Methods:** Blood flow and plasma fibrinolytic factors were measured in both forearms of eight healthy male non-smokers during unilateral brachial artery infusion of the endothelium-dependent vasodilator, substance P (2 to 8 pmol/min). Endothelium-independent vasodilatation was assessed using intra-arterial infusion of sodium nitroprusside (2 to 8 µg/min). Subjects attended after 7 days treatment with transdermal nicotine or placebo in a double blind randomised crossover design. **Results:** Plasma cotinine concentrations rose from  $0.4 \pm 0.1$  (placebo) to  $125 \pm 25$  ng/ml during nicotine administration ( $P < 0.001$ ). On both treatment days, substance P caused dose-dependent increases in blood flow and plasma t-PA antigen and activity concentrations ( $P < 0.001$  for all) but had no effect on plasma plasminogen activator inhibitor type 1 (PAI-1) concentrations. Compared with placebo, nicotine administration increased the substance-P-induced release of t-PA antigen and activity ( $P < 0.05$  for both) without an effect on endothelium-dependent or -independent vasodilatation. **Conclusions:** Short-term transdermal nicotine treatment does not affect endothelium-dependent vasomotion but does increase substance-P-induced t-PA release in vivo in man. This suggests that nicotine administration alters specific aspects of endothelial function and enhances the acute endogenous fibrinolytic capacity in vivo. The long-term effects of nicotine exposure, including the potential to cause depletion of endothelial t-PA stores, now needs to be assessed. © 2001 Elsevier Science B.V. All rights reserved.

**Keywords:** Blood flow; Endothelial function; Thrombolysis

### 1. Introduction

Cigarette smoking is a major risk factor for cardiovascular disease [1] and is associated with acute coronary thrombosis and sudden cardiac death [2]. The mechanisms underlying these associations are unknown although previous studies have demonstrated that regular smokers have impaired endothelium-dependent vasodilatation in both the peripheral [3] and coronary circulations [4]. Endothelial dysfunction may, therefore, contribute to the thrombotic consequences and complications of cigarette smoking. However, whilst endothelium-dependent regulation of vas-

cular tone is important, this may not reflect other crucial aspects of endothelial function such as haemostasis and fibrinolysis.

Focal areas of endothelial denudation and thrombus deposition are a common finding on the surface of atheromatous plaques and usually remain subclinical. However, in the presence of an imbalance in the haemostatic or fibrinolytic system such microthrombi may propagate ultimately leading to arterial occlusion [5]. The degradation and clearance of intravascular thrombus is regulated by tissue-type plasminogen activator (t-PA), a serine protease that is released from the endothelial cells through the translocation of a dynamic intracellular storage pool. The efficacy of plasminogen activation and fibrin degradation is determined by the relative balance between

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the acute release of t-PA and its subsequent inhibition through formation of complexes with its inhibitor, plasminogen activator inhibitor type 1 (PAI-1). Thus, through the acute release of t-PA, endothelial function may have a central role in the regulation of intravascular thrombus formation and, if impaired, may contribute to the pathogenesis of acute myocardial infarction and sudden cardiac death. We have previously described an *in vivo* model to assess the acute release of t-PA from the endothelium in the forearm vascular bed of man [6]. Using this approach, we have been able to demonstrate that cigarette smoking markedly impairs the acute peripheral release of t-PA [7]. However, the mechanism of this attenuated endothelial response was not established.

Nicotine is a major component of cigarette smoke that is rapidly absorbed following inhalation. Nicotine replacement therapy (NRT), either as a gum or patch preparations, has become freely available for over-the-counter sale as a medication to assist in smoking cessation. A number of reports of thrombotic cardiovascular events have been reported amongst individuals regularly using NRT [8]. Cell culture and *in vivo* animal studies [9–12] have indicated that nicotine is directly toxic to endothelial cells and may have an effect on the fibrinolytic balance. There is currently little information about its impact on endothelium-dependent vasodilatation or acute t-PA release *in vivo* in man. Therefore, the aims of this study were to determine whether nicotine impairs endothelium-dependent vasodilatation and whether it affects the endogenous fibrinolytic capacity in the peripheral circulation of man.

## 2. Methods

### 2.1. Subjects

Eight healthy male non-smokers participated in the study which was undertaken with the approval of the local research ethics committee and in accordance with the Declaration of Helsinki. The written informed consent of each subject was obtained before entry into the study. None of the subjects received vasoactive or nonsteroidal anti-inflammatory drugs in the week before the study, and all abstained from alcohol for 24 h before, and from food and caffeine-containing drinks on the day of, the study. All studies were performed in a quiet, temperature-controlled room maintained at 22–25°C.

### 2.2. Intra-arterial drug administration

The brachial artery of the nondominant arm was cannulated with a 27-standard wire gauge steel needle (Cooper's Needle Works Ltd., Sheffield, UK) under local anaesthesia. The cannula was attached to a 16-gauge epidural catheter

(Portex Ltd., Kent, UK) and patency was maintained by infusion of saline (0.9% Baxter Healthcare Ltd., Norfolk, UK) via an IVAC P1000 syringe pump (IVAC Ltd., Basingstoke, UK). The total rate of intra-arterial infusions was maintained constant throughout all studies at 1 ml/min. Pharmaceutical grade substance P (Clinalfa AG, Läufelfingen, Switzerland) and sodium nitroprusside (Faulding DBL Pharmaceutical, Leamington, UK) were administered following dissolution in saline.

### 2.3. Measurements

Blood flow was measured in both forearms by venous occlusion plethysmography using mercury in silastic strain gauges applied to the widest part of the forearm [13]. During measurement periods, the hands were excluded from the circulation by rapid inflation of the wrist cuffs to a pressure of 220 mmHg using E20 Rapid Cuff Inflators (D.E. Hokanson, Washington, USA). Upper arm cuffs were inflated intermittently to 40 mmHg for 10 s in every 15 s to achieve venous occlusion and obtain plethysmographic recordings. Analogue voltage output from an EC-4 Strain Gauge Plethysmograph (D.E. Hokanson) was processed by a MacLab analogue-to-digital converter and Chart™ v3.3.8 software (AD Instruments, Castle Hill, Australia) and recorded onto a Macintosh Classic II computer (Apple Computers, Cupertino, USA). Calibration was achieved using the internal standard of the plethysmograph.

Blood pressure was monitored in the non-infused arm at intervals throughout each study with a semi-automated non-invasive oscillometric sphygmomanometer (Takeda UA 751, Takeda Medical, Tokyo, Japan).

Venous cannulae (17-gauge) were inserted into the deep large subcutaneous veins of the antecubital fossae of both arms. Blood (10 ml) was withdrawn simultaneously from each arm and collected into acidified buffered citrate (Biopool Stabilyte, Umeå, Sweden for t-PA assays) and citrate (Monovette, Sarstedt, Nümbrecht, Germany for PAI-1 assays) tubes, and kept on ice before being centrifuged at 2000×*g* for 30 min at 4°C. Platelet-free plasma was decanted and stored at –80°C before assay. Plasma PAI-1 antigen and t-PA antigen were determined as previously described [6,7] using ELISA (Coaliza PAI-1 and t-PA, Chromogenix AB) and t-PA activity was measured using a photometric method (Coaset t-PA, Chromogenix AB). Haematocrit was determined by an Ac T Counter (Coulter Corporation, Miami, USA) at baseline and during infusion of 8 pmol/min of substance P. Plasma lipid fractions were measured by an enzymatic colorimetric method (Boehringer Mannheim GmbH Diagnostica, Germany). LDL cholesterol was derived according to the method of Friedewald et al. [14]. Plasma cotinine was determined by gas–liquid chromatography as previously described [15].

## 2.4. Study design

Subjects attended on two occasions separated by at least 1 week. Topical nicotine or placebo patches were applied for 7 days prior to the study day in a double blind randomised crossover design. The dose of nicotine administered was 17.5 mg (Nicotinell TTS® 10) but was increased to 35 mg (Nicotinell TTS® 20) for the final 4 days if well tolerated. 'High dose' placebo patches were administered in a similar manner such that double blinding was maintained. On the study day, subjects attended fasted and rested recumbent throughout. Strain gauges and cuffs were applied, and the brachial artery of the non-dominant arm was cannulated as described above. Forearm blood flow was measured every 10 min. Saline was infused for the first 30 min to allow time for equilibration with the final measurement taken as the baseline blood flow, time 0. Thereafter, the subjects received intra-arterial substance P at 2, 4 and 8 pmol/min for 10 min at each dose. After a 30-min washout of saline infusion, the subjects received also an intra-arterial infusion of sodium nitroprusside (SNP) at 2, 4 and 8 µg/min for 10 min at each dose to assess endothelium-independent vasodilatation. Tissue-plasminogen activator was measured at baseline and with each dose of substance P. Plasminogen activator inhibitor was measured at baseline and at substance P 8 pmol/min. In this model, SNP infusion does not affect fibrinolytic variables [6,16] and was, therefore, not measured. The order of the substance P and SNP infusions was randomised between subjects in each study but remained constant for each subject. Plasma cotinine concentrations were measured at baseline on each study day.

## 2.5. Data analysis and statistics

Plethysmographic data were extracted from the Chart™ data files and forearm blood flow (FBF) was calculated for individual venous occlusion cuff inflations by use of a template spreadsheet (Excel v5.0; Microsoft Corporation, Cambridge, USA). Recordings from the first 60 s after wrist cuff inflation were not used because of the variability in blood flow this causes [13]. The average of the last five recordings from a 3-min interval of FBF measurement were used for analysis. The percentage change from baseline blood flow in the infused arm was calculated, as previously described [13], from the ratio of the infused and non-infused arm blood flow:

$$\% \text{ change in blood flow} = 100 \times \frac{(I_t/NI_t - I_b/NI_b)}{I_b/NI_b}$$

where  $I_b$  and  $NI_b$  are the infused and non-infused forearm blood flows at baseline (time 0) respectively, and  $I_t$  and  $NI_t$  are the infused and non-infused forearm blood flows at a given time point, respectively.

Estimated net release of t-PA activity and antigen was previously defined [6,7] as the product of the infused forearm plasma flow (based on the mean haematocrit, Hct, and the infused forearm blood flow, FBF) and the concentration difference between the infused ( $[t\text{-PA}]_{\text{Inf}}$ ) and non-infused arms ( $[t\text{-PA}]_{\text{Non-inf}}$ )

$$\text{estimated net t-PA release} = \text{FBF} \times \{1 - \text{Hct}\} \times \{[t\text{-PA}]_{\text{Inf}} - [t\text{-PA}]_{\text{Non-inf}}\}.$$

Data were examined, where appropriate, by two-way analysis of variance (ANOVA) with repeated measures and two-tailed paired Student's *t*-test using Excel v5.0 (Microsoft). All results are expressed as mean ± standard error of the mean (S.E.M.). Statistical significance was taken at the 5% level.

## 3. Results

All subjects were normotensive with a normal serum lipid profile and plasma glucose concentration (Table 1). Nicotine administration significantly increased plasma cotinine levels from  $0.4 \pm 0.1$  to  $125 \pm 25$  ng/ml ( $P < 0.001$ , paired *t*-test). There were no significant changes in blood pressure, heart rate, haematocrit or blood flow in the non-infused forearm during each of the study days (Table 2). During nicotine administration, the heart rate and the non-infused and infused forearm blood flows were consistently higher than during placebo (Table 2). Two subjects were unable to tolerate the higher dose of nicotine and returned to the 17.5 mg nicotine patch for the remainder of the 7 days.

Substance P and SNP caused dose-dependent increases in FBF during placebo and nicotine administration ( $P < 0.001$  for all, ANOVA). Because of baseline differences, absolute FBF during substance P and SNP infusion were greater with nicotine administration ( $P < 0.05$ , two-way ANOVA). However taking account of these baseline differences, there were no significant differences in the percentage increase in FBF with substance P or SNP infusion during nicotine administration (Table 2).

Table 1  
Baseline subject characteristics (mean ± S.E.M.)

Age	29 ± 3	years
Heart rate	68 ± 3	/min
Mean arterial pressure	91 ± 3	mmHg
Fasting plasma glucose concentration	87 ± 2	mg/dl
Serum lipid profile		
Total cholesterol concentration	154 ± 8	mg/dl
HDL cholesterol concentration	46 ± 4	mg/dl
LDL cholesterol concentration	77 ± 8	mg/dl
Triglyceride concentration	114 ± 26	mg/dl

Table 2

Forearm blood flow and systemic haemodynamics during nicotine and placebo administration

	Substance P (pmol/min)				Sodium nitroprusside ( $\mu$ g/min)			
	0	2	4	8	0	2	4	8
<b>Nicotine administration</b>								
Heart rate (/min)	69 $\pm$ 3	69 $\pm$ 3	67 $\pm$ 3	71 $\pm$ 3	71 $\pm$ 3	70 $\pm$ 3	73 $\pm$ 5	73 $\pm$ 4 <sup>†</sup>
Mean arterial pressure (mmHg)	92 $\pm$ 4	92 $\pm$ 4	94 $\pm$ 4	92 $\pm$ 3	95 $\pm$ 3	94 $\pm$ 4	96 $\pm$ 4	95 $\pm$ 4
Absolute forearm blood flow (ml/100 ml/min)								
Non-infused arm	4.8 $\pm$ 0.7	5.2 $\pm$ 0.8	5.1 $\pm$ 0.8	5.2 $\pm$ 0.9	5.3 $\pm$ 0.9	5.1 $\pm$ 0.9	5.6 $\pm$ 1	4.8 $\pm$ 0.8* <sup>†</sup>
Infused arm	5 $\pm$ 0.6	13 $\pm$ 0.7	15.5 $\pm$ 0.9	18.7 $\pm$ 1.3*	5.2 $\pm$ 0.8	11.2 $\pm$ 0.9	15 $\pm$ 1.4	18 $\pm$ 1.7* <sup>†</sup>
Percentage change in forearm blood flow	0	159 $\pm$ 25	217 $\pm$ 34	281 $\pm$ 46*	0	164 $\pm$ 40	227 $\pm$ 55	368 $\pm$ 80*
<b>Placebo administration</b>								
Heart rate (/min)	68 $\pm$ 3	63 $\pm$ 2	62 $\pm$ 1	68 $\pm$ 3	68 $\pm$ 3	69 $\pm$ 2	66 $\pm$ 1	71 $\pm$ 2 <sup>†</sup>
Mean arterial pressure (mmHg)	90 $\pm$ 3	91 $\pm$ 3	92 $\pm$ 2	92 $\pm$ 2	95 $\pm$ 2	93 $\pm$ 3	92 $\pm$ 3	91 $\pm$ 2
Absolute forearm blood flow (ml/100 ml/min)								
Non-infused arm	3.5 $\pm$ 0.5	3.8 $\pm$ 0.5	3.5 $\pm$ 0.6	4 $\pm$ 0.5	4.1 $\pm$ 0.6	3.8 $\pm$ 0.5	3.5 $\pm$ 0.4	3.4 $\pm$ 0.3* <sup>†</sup>
Infused arm	3.9 $\pm$ 0.8	11 $\pm$ 0.9	12.3 $\pm$ 1.1	15.2 $\pm$ 1*	4.3 $\pm$ 0.7	10.5 $\pm$ 0.7	12 $\pm$ 1.2	15.4 $\pm$ 1.3* <sup>†</sup>
Percentage change in forearm blood flow	0	211 $\pm$ 44	300 $\pm$ 66	309 $\pm$ 52*	0	178 $\pm$ 23	242 $\pm$ 30	337 $\pm$ 45*

\* $P < 0.001$  one-way ANOVA; <sup>†</sup> $P < 0.05$  two-way ANOVA (nicotine vs. placebo administration).

Compared with the non-infused arm, substance P caused dose-dependent increases in plasma concentration of t-PA antigen and activity in the infused arm during nicotine and placebo administration (Table 3). The infused arm plasma concentrations and estimated net release of t-PA antigen and activity were greater with nicotine administration in comparison to placebo (Fig. 1 and Table 3). There were no significant changes in plasma PAI-1 concentrations either between treatment groups or before and after substance P infusion (Table 3).

#### 4. Discussion

In healthy male volunteers, short-term nicotine administration, sufficient to increase plasma cotinine concentrations to those observed in cigarette smokers [3,17], was associated with an increase in substance-P-induced t-PA release, but had no effect on endothelium-dependent vasodilatation. This suggests that short-term nicotine administration alters specific aspects of endothelial function and enhances the acute endogenous fibrinolytic capacity in

Table 3

Plasma tissue plasminogen activator (t-PA) and plasminogen activator inhibitor type 1 (PAI 1) concentrations during nicotine and placebo administration

Substance P dose (pmol/min)	Nicotine				Placebo			
	0	2	4	8	0	2	4	8
<b>Plasma t-PA antigen (ng/ml)</b>								
Non-infused arm	4.5 $\pm$ 0.3	4.4 $\pm$ 0.2	5.0 $\pm$ 0.5	4.5 $\pm$ 0.2	4.2 $\pm$ 0.3	4.2 $\pm$ 0.4	4.2 $\pm$ 0.4	4.4 $\pm$ 0.5
Infused arm	3.9 $\pm$ 0.3	4.3 $\pm$ 0.3	4.9 $\pm$ 0.2	6.4 $\pm$ 0.6*	4.1 $\pm$ 0.4	4.6 $\pm$ 0.4	4.8 $\pm$ 0.4	5.8 $\pm$ 0.7*
Forearm difference	-0.6 $\pm$ 0.2	-0.1 $\pm$ 0.3	0.1 $\pm$ 0.4	1.9 $\pm$ 0.6 <sup>†</sup>	-0.1 $\pm$ 0.1	0.4 $\pm$ 0.1	0.6 $\pm$ 0.2	1.4 $\pm$ 0.4 <sup>†</sup>
<b>Plasma t-PA activity (IU/ml)</b>								
Non-infused arm	1.9 $\pm$ 0.1	2.2 $\pm$ 0.2	2.3 $\pm$ 0.2	2.3 $\pm$ 0.2	1.9 $\pm$ 0.1	2.0 $\pm$ 0.2	2.1 $\pm$ 0.2	2.3 $\pm$ 0.2
Infused arm	1.9 $\pm$ 0.1	2.4 $\pm$ 0.2	3.8 $\pm$ 0.7	7.1 $\pm$ 1.7*	1.8 $\pm$ 0.1	2.2 $\pm$ 0.2	2.8 $\pm$ 0.5	4.0 $\pm$ 0.7* <sup>†</sup>
Forearm difference	0.0 $\pm$ 0.1	0.2 $\pm$ 0.1	1.5 $\pm$ 0.5	4.8 $\pm$ 1.6 <sup>†</sup>	0.0 $\pm$ 0.1	0.1 $\pm$ 0.2	0.7 $\pm$ 0.5	1.7 $\pm$ 0.6 <sup>††</sup>
<b>Plasma PAI-1 antigen (ng/ml)</b>								
Non-infused arm	32 $\pm$ 6	—	—	30 $\pm$ 6	35 $\pm$ 8	—	—	40 $\pm$ 6
Infused arm	30 $\pm$ 6	—	—	31 $\pm$ 5	37 $\pm$ 9	—	—	34 $\pm$ 8

\* $P < 0.05$  two-way ANOVA (infused vs. non-infused arm); <sup>†</sup> $P < 0.05$  two-way ANOVA (nicotine vs. placebo); <sup>††</sup> $P < 0.001$  one-way ANOVA.



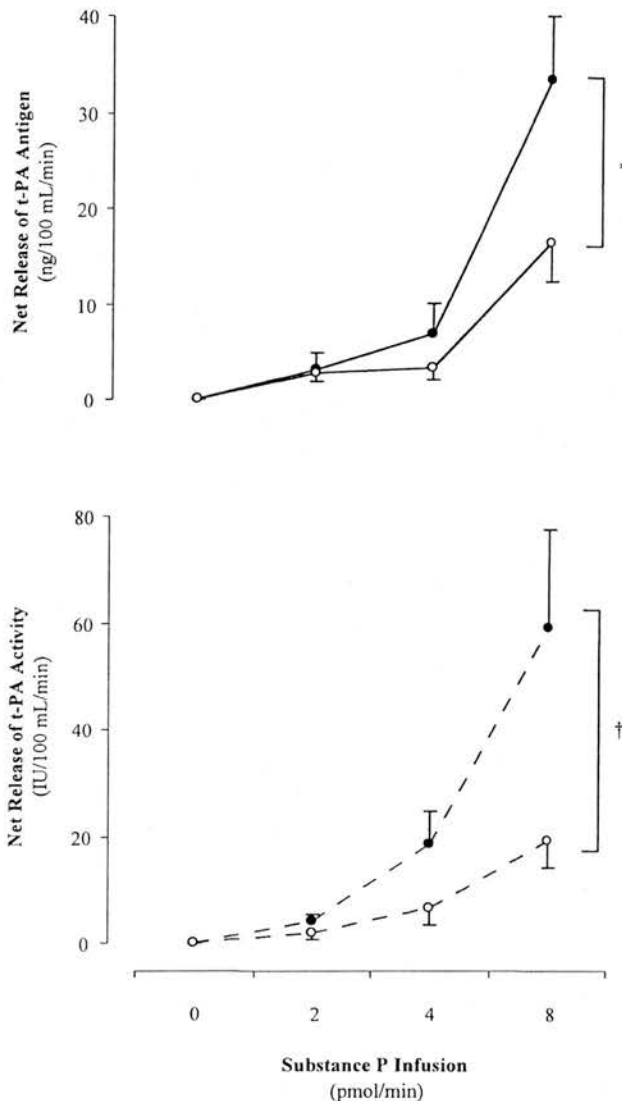


Fig. 1. Net release of tissue plasminogen activator (t-PA) antigen (solid lines) and activity (dashed lines) during placebo (open circles) and nicotine (closed circles) administration. One-way ANOVA:  $P < 0.001$  for all responses. Two-way ANOVA: \* $P < 0.05$ ; † $P < 0.001$  (nicotine vs. placebo).

vivo. The long-term effects of nicotine exposure, including the potential to cause depletion of endothelial t-PA stores, now needs to be assessed, particularly in recent ex-smokers.

There are many potentially toxic chemicals in cigarette smoke but there has been continued concern that nicotine may have direct injurious effects, especially given anecdotal reports of thrombotic cardiovascular events amongst individuals regularly using NRT [8]. Although species differences exist, *in vitro* studies have shown that acute nicotine administration increases t-PA release from bovine aortic endothelial cells [11] while *in vivo* rodent studies

suggest chronic nicotine exposure depletes the free pool of brain capillary t-PA antigen [12]. The act of smoking acutely releases t-PA causing an elevation in plasma t-PA concentrations [18] that appears to be blunted in chronic cigarette smokers [19]. Indeed, chronic smoking causes a reduction in the subsequent ability to release t-PA when challenged with desmopressin [20] or venous occlusion [21]. These findings are consistent with our observations of enhanced acute t-PA release with short-term nicotine exposure and our previous findings [7] of increased basal plasma t-PA concentrations in chronic cigarette smokers but a reduced acute release of t-PA. However, further studies are now required to assess how rapidly the impairment of t-PA release reverses on cessation of cigarette smoking, and what effect nicotine supplementation has in recent ex-smokers.

The mechanisms of action of nicotine on t-PA release are unknown, but may be mediated by induction of gene expression [11] or processing of t-PA antigen at the post-transcriptional level [12]. As with cigarette smoking, there does appear to be an important difference between the effects of nicotine during acute and chronic administration [11,12], and whilst short-term nicotine administration appears to potentiate acute t-PA release, chronic administration may cause a reduction. This may be a consequence of the depletion of intracellular t-PA stores, desensitisation of the endothelium to further stimulation, or paradoxically a reduction in the acute storage pool due to preferential up-regulation of constitutive t-PA release. In the present investigation, short-term nicotine exposure significantly increased the capacity of the endothelium to release t-PA when challenged by substance P infusion. However, we did not observe a rise in basal plasma concentration of t-PA antigen to accompany this associated increase in acute stimulated t-PA release. This suggests that nicotine may require a more protracted period of administration or necessitate synergy with a stimulus for t-PA release, such as other gas-phase components of cigarette smoke.

Epidemiological studies of long-term nicotine treatment have not been conducted but relevant information can be inferred from studies of chewing tobacco or snuff users where there is systemic absorption of nicotine but not of other combustion products. These studies have found no evidence of an increased risk of myocardial infarction or sudden cardiac death [22,23]. Two prospective studies involving treatment of smokers with known cardiovascular disease using transdermal nicotine have also failed to find an association between nicotine use and the risk of acute cardiovascular events [24,25]. Indeed, one study of smokers [25] reported more adverse events with placebo than nicotine patches suggesting a protective role of nicotine. Moreover, an experimental study of smokers with known coronary artery disease demonstrated that nicotine supplementation caused a substantial reduction in exercise-induced reversible myocardial perfusion defects as assessed

by quantitative SPECT scanning [26]. Although there are differences between epidemiological and experimental studies, these results suggest that nicotine in itself is not a direct cause of tobacco-related cardiovascular disease.

In contrast to previous *in vivo* studies [27–29], we did not find an attenuation in blood flow responses to the endothelium-dependent vasodilatation during nicotine administration. This disparity may be due to species differences [27] or to the study populations investigated [29]. Sarabi et al. [29] assessed the effects of nicotine supplementation in habitual smokers and reported a difference in a derived index of endothelial function. However, no direct comparisons were made of the blood flow responses to methacholine. In addition, nicotine treatment was associated with a change in systemic blood pressure and the authors conceded that this may have accounted for their findings. These contrasting observations may also be explained by the differing signal transduction pathways involved in muscarinic and substance-P-induced vasodilatation [30,31]. Indeed, previous studies assessing endothelial function, using both substance P and acetylcholine administration, have documented either concordant [32,33] or discordant [34,35] responses that are, in part, dependent upon the disease processes under investigation. Moreover, we chose to use substance P as an endothelial cell stimulant because acetylcholine does not induce the acute release of t-PA in the human forearm [36]. These observations highlight the important differences between the various agents used to stimulate the endothelium as well as the differing manifestations of endothelial dysfunction that are dependent on the nature of the underlying cellular injury.

Another finding of the present study is that nicotine administration was associated with an increase in basal forearm blood flow suggesting a vasodilator effect of nicotine on the peripheral vascular bed. The cardiovascular effects of systemic nicotine administration are complex and include actions of nicotine on the central and peripheral nervous systems. Of the studies that have examined the direct effect of nicotine on vascular reactivity, all have suggested a vasodilator action. *Ex vivo* studies indicate that nicotine induces a neurally and nitric oxide-mediated, endothelium-independent, vasodilatation [37,38]. This has been confirmed by an *in vivo* canine model [39] that demonstrated that the intracarotid injection of nicotine increased cerebral blood flow through the release of nitric oxide from vagal nerve terminals. Moreover, Fewings et al. [40] have reported that intra-arterial nicotine infusion increases forearm blood flow in man. Our findings suggest that short-term nicotine administration does not impair endothelium-dependent vasomotion but does cause systemic vasodilatation as indicated by an elevation in resting forearm blood flow and heart rate.

In conclusion, we have demonstrated, for the first time, that nicotine administration alters specific aspects of endothelial function and enhances the acute endogenous

fibrinolytic capacity *in vivo*. Future studies are now needed to assess the effects of long-term nicotine exposure, including the potential to cause depletion of endothelial t-PA stores, particularly in recent ex-smokers.

## Acknowledgements

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**PUBLICATION 14**

## CARDIOVASCULAR MEDICINE

# Hypercholesterolaemia and lipid lowering treatment do not affect the acute endogenous fibrinolytic capacity in vivo

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**Objective:** To assess acute tissue plasminogen activator (t-PA) release in vivo in patients with hypercholesterolaemia in the presence and absence of lipid lowering treatment and in matched normocholesterolaemic controls.

**Design:** Parallel group comparison and double blind randomised crossover.

**Setting:** University hospital.

**Patients:** Eight patients with hypercholesterolaemia ( $> 7.8$  mmol/l) and eight matched normocholesterolaemic controls ( $< 5.5$  mmol/l).

**Methods:** Blood flow and plasma fibrinolytic factors were measured in both forearms during unilateral brachial artery infusions of the endothelium dependent vasodilator substance P (2–8 pmol/min) and the endothelium independent vasodilator sodium nitroprusside (1–4 µg/min).

**Interventions:** In patients, measurements were made on three occasions: at baseline and after six weeks of placebo or pravastatin 40 mg daily administered in a double blind randomised crossover design.

**Main outcome measures:** Acute release of t-PA.

**Results:** Compared with patients, in normocholesterolaemic control subjects substance P caused greater dose dependent increases in forearm blood flow ( $p < 0.05$ ) but similar increases in plasma t-PA antigen and activity concentrations. During pravastatin treatment in patients, total serum cholesterol fell by 22% from a mean (SEM) of 8.1 (0.3) to 6.4 (0.4) mmol/l ( $p = 0.002$ ) and substance P induced vasodilatation was no longer significantly impaired in comparison with controls. However, despite reproducible responses, pravastatin treatment was not associated with significant changes in basal or substance P induced t-PA release.

**Conclusions:** Hypercholesterolaemia and lipid lowering treatment cause no demonstrable effects on acute substance P induced t-PA release in vivo. This suggests that the preventative benefits of lipid lowering treatment are unlikely to be mediated by improvements in endogenous fibrinolysis.

Hypercholesterolaemia impairs endothelial cell function,<sup>1-3</sup> predisposes vessels to damage, and contributes to vascular occlusion.<sup>4</sup> Previous studies have shown that endothelium dependent nitric oxide mediated vasodilatation is impaired in patients with hypercholesterolaemia,<sup>1-3</sup> an effect that is reversed by lipid lowering treatment.<sup>5-7</sup> Although other endothelial cell markers have been described,<sup>8-9</sup> the fibrinolytic factor tissue plasminogen activator (t-PA) and its inhibitor, plasminogen activator inhibitor type 1 (PAI-1), are potentially important markers that are intimately linked to the risk of atherothrombosis.<sup>10-11</sup>

Endothelial cells in the precapillary arterioles and postcapillary venules<sup>12</sup> synthesise and release t-PA and PAI-1 both basally and in response to various coagulation factors and stimulants. The rapid mobilisation of t-PA from the endothelium is crucial if endogenous fibrinolysis within the arterial circulation is to be effective, with thrombus dissolution being much more effective if t-PA is incorporated during, rather than after, thrombus formation.<sup>13-14</sup> The importance of endogenous t-PA release is exemplified by the high rate of spontaneous reperfusion in the infarct related artery after acute myocardial infarction, which occurs in around 30% of patients within the first 12 hours.<sup>15</sup>

Epidemiological studies examining total plasma t-PA and PAI-1 concentrations in patients with ischaemic heart disease have observed a positive correlation with coronary events.<sup>10-11</sup> It would be anticipated, however, that high t-PA concentra-

tions would protect against subsequent coronary events rather than the reverse. This paradoxical association is, in part, explained by the concomitant increase of PAI-1, which complexes with t-PA and therefore causes an overall reduction in free t-PA "activity".<sup>16-17</sup> It is this free and unbound t-PA that is physiologically active and central to endogenous fibrinolysis. However, the capacity of endothelial cells to release t-PA from intracellular storage pools and the rapidity with which this can be mobilised may not necessarily be reflected in the basal circulating plasma concentrations of t-PA antigen or its activity.<sup>18-19</sup> We have recently described an in vivo model to assess the acute release of t-PA in the forearm of humans.<sup>20</sup> Using intrabrachial infusions of substance P, we have shown a dose dependent release of t-PA without causing a significant release in PAI-1. Moreover, we have also reported<sup>21</sup> that t-PA release is inhibited by nitric oxide synthase inhibition with L-N<sup>G</sup>-monomethylarginine, suggesting that endothelial dysfunction may impair the release of t-PA.

Given that substance P induced vasodilatation has been reported to be impaired in patients with

**Abbreviations:** ELISA, enzyme linked immunosorbent assay; FBF, forearm blood flow; Hct, Haematocrit; LDL, low density lipoprotein; PAI-1, plasminogen activator inhibitor type 1; t-PA, tissue plasminogen activator; WOSCOPS, west of Scotland coronary prevention study



hypercholesterolaemia,<sup>1</sup> the aims of the present study were to determine whether there is also an impairment of t-PA release in patients with hypercholesterolaemia and whether treatment with pravastatin<sup>3</sup> could enhance t-PA release in these patients.

## METHODS

### Patients and control subjects

Eight patients with primary hypercholesterolaemia were recruited from the clinic if their serum cholesterol concentrations exceeded 7.8 mmol/l (> 296 mg/dl). Following screening with clinical examination, repeated questioning for symptoms, clinical chemistry screen (liver enzymes, electrolytes, urea, and creatine), haematology screen (full blood and differential counts), urinalysis, and 12 lead ECG, patients were excluded if they had diabetes mellitus, hypertension, ischaemic heart disease, peripheral vascular disease, an abnormal resting ECG, or other clinically significant disease. Patients were matched with normocholesterolaemic (< 5.5 mmol/l; < 209 mg/dl) healthy control subjects for age, sex, and smoking habit. All studies were undertaken with the approval of the local research ethics committee and in accordance with the Declaration of Helsinki. The written informed consent of each subject was obtained before entry into the study. None of the subjects was receiving vasoactive or non-steroidal anti-inflammatory drugs in the week before each phase of the study, and all abstained from alcohol for 24 hours and from food, tobacco, and drinks containing caffeine for at least nine hours before each study. All studies were performed in a quiet temperature controlled room maintained at 23.5–24.5°C.

### Intra-arterial administration and drugs

The brachial artery of the non-dominant arm was cannulated with a 27 standard wire gauge steel needle (Cooper's Needle Works Ltd, Birmingham, UK) under 1% lignocaine (Xylocaine; Astra Pharmaceuticals Ltd, Kings Langley, UK) local anaesthesia. The cannula was attached to a 16 gauge epidural catheter (Portex Ltd, Hythe, UK) and patency was maintained by infusion of saline (0.9%; Baxter Healthcare Ltd, Thetford, UK) through an IVAC P1000 syringe pump (IVAC Ltd, Basingstoke, UK). The total rate of intra-arterial infusions was maintained constant throughout all studies at 1 ml/min. Pharmaceutical grade substance P (Clinalfa AG, Läufelfingen, Switzerland) and sodium nitroprusside (David Bull Laboratories, Warwick, UK) were administered following dissolution in saline.

### Forearm blood flow and blood pressure

Blood flow was measured in both forearms by venous occlusion plethysmography using mercury in silastic strain gauges applied to the widest part of the forearm.<sup>22</sup> During measurement periods the hands were excluded from the circulation by rapid inflation of the wrist cuffs to a pressure of 220 mm Hg using E20 rapid cuff inflators (DE Hokanson Inc, Bellevue, Washington, USA). Upper arm cuffs were inflated intermittently to 40 mm Hg for 10 s in every 15 s to achieve venous occlusion and obtain plethysmographic recordings. Analogue voltage output from an EC-4 strain gauge plethysmograph (DE Hokanson) was processed by a MacLab analogue to digital converter and Chart version 3.3.8 software (AD Instruments Ltd, Castle Hill, Australia) and recorded on to a Macintosh Classic II computer (Apple Computers Inc, Cupertino, California USA). Calibration was achieved using the internal standard of the plethysmograph.

Blood pressure was monitored in the non-infused arm at intervals throughout each study using a semiautomated non-invasive oscillometric sphygmomanometer (Takeda UA 751, Takeda Medical Inc, Tokyo, Japan).<sup>23</sup>

### Venous sampling and assays

Venous cannulae (17 gauge) were inserted into large subcutaneous veins of the antecubital fossa in both arms. Ten

millilitres of blood was withdrawn simultaneously from each arm and collected into acidified buffered citrate (Biopool Stability, Umeå, Sweden, for t-PA assays) and citrate (Monovette, Sarstedt, Nümbrecht, Germany, for PAI-1 assays) tubes, and kept on ice before being centrifuged at 2000 *g* for 30 minutes at 4°C. Platelet free plasma was decanted and stored at –80°C before assay.<sup>24</sup>

Plasma t-PA and PAI-1 antigen concentrations were determined using an enzyme linked immunosorbent assay (ELISA): Coaliza t-PA<sup>25</sup> and Coaliza PAI-1<sup>26</sup> (Chromogenix AB, Mölndal, Sweden), respectively. Plasma t-PA and PAI-1 activities were determined by a photometric method: Coaset t-PA and Coatest PAI-1 (Chromogenix AB), respectively.<sup>27</sup> Plasma PAI-1 activity is determined by a back titration method and represents the total t-PA inhibitory activity of the plasma.<sup>27</sup> All fibrinolytic assays were performed in duplicate and the mean value was taken. Intra-assay coefficients of variation were 7.0% and 5.5% for t-PA and PAI-1 antigen and 4.0% and 2.4% for activity, respectively. Interassay coefficients of variability were 4.0%, 7.3%, 4.0% and 7.6%, respectively. The limits of sensitivity of the assays were 0.5 ng/ml, 2.5 ng/ml, 0.10 IU/ml, and 5 AU/ml, respectively. Haematocrit (Hct) was determined by capillary tube centrifugation of blood anticoagulated by ethylene diamine tetra acetic acid and was obtained from the infused forearm at baseline and at 8 pmol/min of substance P. Serum cholesterol and triglyceride concentrations were determined by an enzymatic colorimetric method (Boehringer Mannheim GmbH Diagnostica, Mannheim, Germany). Low density lipoprotein (LDL) cholesterol was determined by the method of Friedewald and colleagues.<sup>28</sup>

### Study design

All patients attended on each of the three study days: baseline and following six weeks' treatment with placebo and six weeks' treatment with pravastatin 40 mg daily. Placebo and pravastatin treatments were given in a randomised double blind crossover design. Control subjects attended on one occasion only.

On each study day, subjects attended fasted at 0900 and rested recumbent throughout. Strain gauges and cuffs were applied and the brachial artery of the non-dominant arm was cannulated. Throughout all protocols, forearm blood flow (FBF) was measured every 10 minutes. Before administration of substance P and sodium nitroprusside, saline was infused for 30 minutes to allow time for equilibration and the final blood flow measurement during saline infusion was taken as the basal FBF. Substance P was infused at 2, 4, and 8 pmol/min for 10 minutes at each dose<sup>19–21</sup> and sodium nitroprusside was infused at 1, 2, and 4 µg/min for 10 minutes at each dose.<sup>20</sup> The order of administration of substance P and sodium nitroprusside was randomised.

### Data analysis and statistics

Plethysmographic data were extracted from the Chart data files and FBFs were calculated for individual venous occlusion cuff inflations by use of a template spreadsheet (Excel version 5.0; Microsoft Corporation, Redmond, Washington, USA). Recordings from the first 60 seconds after wrist cuff inflation were not used because of the variability in blood flow that this causes.<sup>22</sup> Usually the last five flow recordings in each three minute measurement period were calculated and averaged for each arm. To reduce the variability of blood flow data, the ratio of flows in the two arms was calculated for each time point, in effect using the non-infused arm as a contemporaneous control for the infused arm.<sup>22</sup> Percentage changes in the infused FBF were calculated<sup>22</sup> as follows:

$$\% \text{ change in blood flow} = 100 \times (I_i/NI_i - I_0/NI_0) / I_0/NI_0$$

where  $I_0$  and  $Ni_0$  are the infused and non-infused FBFs at baseline (time 0), respectively, and  $I_i$  and  $Ni_i$  are the infused and non-infused FBFs at a given time point, respectively.

**Table 1** Lipid profile, haemodynamics, absolute forearm blood flow, and haematocrit at baseline and during placebo and pravastatin treatment

	Controls	Patients		
		Baseline	Placebo	Pravastatin
Age (years)	43 (3)	46 (3)	—	—
Sex (male:female)	5:3	5:3	—	—
Body mass index (kg/m <sup>2</sup> )	24 (1)	25 (1)	—	—
Smokers	3	3	—	—
Total cholesterol (mmol/l)	5.0 (0.2)***	8.1 (0.3)	8.2 (0.3)	6.4 (0.4)**
LDL cholesterol (mmol/l)	3.2 (0.2)***	5.8 (0.5)	6.1 (0.5)	4.3 (0.4)*
HDL cholesterol (mmol/l)	1.1 (0.1)	1.1 (0.1)	1.1 (0.2)	1.2 (0.2)
Triglycerides (mmol/l)	1.5 (0.4)	2.7 (0.4)	2.3 (0.4)	1.9 (0.3)
Mean arterial pressure (mm Hg)	88 (5)	87 (2)	87 (4)	87 (2)
Heart rate (beats/min)	64 (2)	62 (3)	63 (5)	62 (4)
Basal blood flow (mL/100 mL/min)				
Non-infused arm	3.0 (0.5)	2.4 (0.3)	2.9 (0.3)	2.4 (0.3)
Infused arm	3.7 (0.7)	3.6 (0.3)	3.2 (0.3)	2.5 (0.3)
Haematocrit	0.39 (0.01)	0.43 (0.01)	0.42 (0.01)	0.41 (0.01)

Data are mean (SEM). Unpaired *t* test \*\*\**p* < 0.001 (controls versus patients at baseline); \**p* = 0.04, \*\**p* = 0.003 (patients: pravastatin versus controls); Paired *t* test \**p* = 0.02, \*\**p* < 0.003 (patients: placebo versus pravastatin); Analysis of variance \**p* = 0.04, \*\**p* < 0.002 (three way analysis of variance for patient groups). HDL, high density lipoprotein; LDL, low density lipoprotein.

Estimated net release of t-PA activity and antigen was defined previously<sup>19,21</sup> as the product of the infused forearm plasma flow (based on the mean Hct and the infused FBF) and the concentration difference between the infused ([t-PA]<sub>inf</sub>) and non-infused arms ([t-PA]<sub>non-inf</sub>):

$$\text{estimated net t-PA release} = \text{FBF} \times (1 - \text{Hct}) \times ([\text{t-PA}]_{\text{inf}} - [\text{t-PA}]_{\text{non-inf}})$$

Data were examined, where appropriate, by two way analysis of variance with repeated measures and two tailed paired Student's *t* test using Excel version 5.0. Reproducibility of the responses to substance P infusion was assessed by comparing the baseline data with placebo treatment study days using the method of Bland and Altman.<sup>20</sup> Coefficients of reproducibility were determined for 95% confidence intervals using the Student's *t* distribution. All results are expressed as mean (SEM). Significance was taken at the 5% level. On the basis of previous data<sup>20</sup> the study had 90% power to detect a 20% change in plasma t-PA concentrations between treatment periods at the 5% level.

## RESULTS

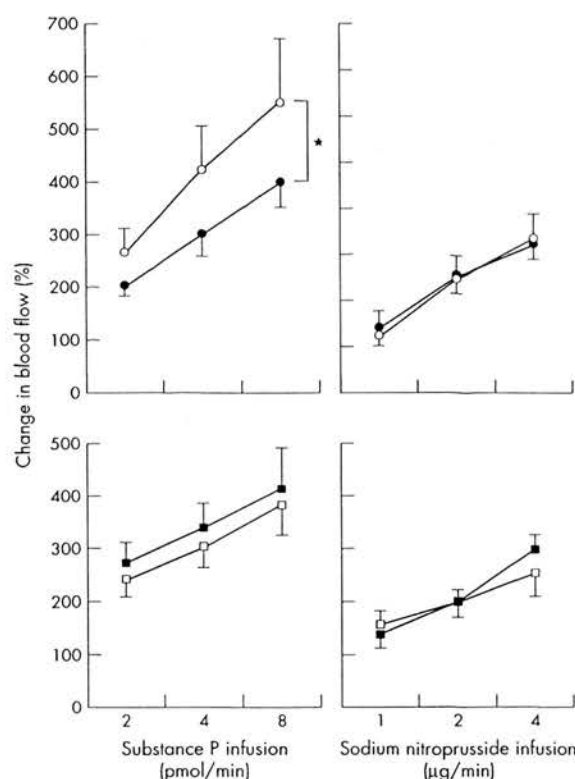
Table 1 shows patient and control subject characteristics. The groups were well matched for age, sex, body mass index, smoking habit, blood pressure, heart rate, Hct, and FBF. Total cholesterol and LDL cholesterol concentrations were significantly higher in the patient group and fell during pravastatin but not placebo treatment (table 1). On each of the study days in the patient and control groups, blood pressure, heart rate, Hct, blood flow, and t-PA concentrations of the non-infused forearm did not change.

### Blood flow responses

Substance P caused dose dependent increases in blood flow of the infused forearm in both patients and controls (*p* < 0.001 for both, analysis of variance; fig 1). In comparison to the control group, the substance P induced increases in blood flow were significantly less in the patient group (*p* < 0.05, two way analysis of variance; fig 1). During pravastatin treatment, substance P appeared to cause a slightly greater increase in blood flow but this was not significantly different from baseline responses (*p* = 0.30, two way analysis of variance, baseline

versus pravastatin) although it was no longer significantly different from control group responses (*p* = 0.24, two way analysis of variance).

Sodium nitroprusside also caused dose dependent increases in blood flow of the infused forearm (*p* < 0.001, analysis of



**Figure 1** Forearm blood flow responses to substance P and sodium nitroprusside administration in control subjects (open circles, upper panels) and patients at baseline (closed circles, upper panels), and in patients during placebo (open squares, lower panels) and during pravastatin treatment (closed squares, lower panels). *p* < 0.001 for each forearm blood flow response (one way analysis of variance). \**p* < 0.05 (two way analysis of variance, controls versus patients).

**Table 2** Plasma tissue plasminogen activator (t-PA) antigen and activity in patients at baseline and during placebo and pravastatin treatment and in a matched control group

		Substance P dose (pmol/min)	Controls	Patients		
				Baseline	Placebo	Pravastatin
t-PA antigen (ng/ml)						
Infused forearm*		0	5.0 (0.5)	4.6 (0.8)	4.7 (0.6)	4.5 (0.6)
		2	5.6 (0.7)	5.4 (0.8)	5.4 (0.6)	5.0 (0.4)
		4	7.4 (0.8)	5.7 (0.9)	6.0 (0.9)	5.8 (0.6)
		8	8.0 (1.3)	7.4 (1.1)	7.5 (1.3)	7.6 (1.1)
Non-infused forearm		0	4.7 (0.5)	4.8 (0.7)	4.5 (0.6)	4.6 (0.6)
		2	4.6 (0.4)	4.8 (0.7)	4.5 (0.5)	4.5 (0.6)
		4	5.8 (0.6)	5.1 (0.8)	4.5 (0.8)	4.6 (0.6)
		8	5.7 (0.6)	5.0 (0.8)	5.1 (0.6)	4.8 (0.6)
t-PA activity (IU/ml)						
Infused forearm*		0	0.6 (0.1)	0.5 (0.1)	0.6 (0.1)	0.5 (0.1)
		2	1.7 (0.5)	1.3 (0.5)	1.1 (0.4)	1.6 (0.8)
		4	2.7 (1.0)	2.0 (0.6)	1.7 (0.9)	2.2 (1.1)
		8	4.5 (1.5)	3.4 (1.1)	3.9 (1.4)	3.1 (1.2)
Non-infused forearm		0	0.7 (0.1)	0.4 (0.1)	0.5 (0.1)	0.4 (0.1)
		2	0.8 (0.1)	0.5 (0.1)	0.6 (0.2)	0.5 (0.1)
		4	1.1 (0.2)	0.6 (0.1)	0.7 (0.2)	0.6 (0.1)
		8	1.7 (0.5)	0.7 (0.2)	0.8 (0.2)	0.7 (0.2)

Data are mean (SEM). \* $p < 0.001$ , one way analysis of variance in the infused forearm for each group.

variance; fig 1), which were similar in patient and control groups. Neither pravastatin nor placebo treatment influenced the response to sodium nitroprusside infusion.

#### Fibrinolytic factor responses

Substance P caused dose dependent increases in plasma t-PA antigen and activity concentrations in the infused forearm of both patients and controls ( $p < 0.001$  for both, analysis of variance; table 2). Substance P induced increases in plasma t-PA concentrations were similar in both groups and, in the patient group, were unaffected by pravastatin or placebo treatment. The concentration differences between the forearms and the estimated net release of t-PA antigen and activity also increased dose dependently ( $p < 0.001$ , analysis of variance; fig 2), which did not differ between the patient and control groups or during pravastatin and placebo treatment. There were no significant differences in plasma PAI-1 antigen and activity concentrations at baseline, although there appeared to be a trend for plasma PAI-1 antigen concentrations to be lower in the control group ( $p > 0.12$ , unpaired  $t$  test; table 3). Plasma PAI-1 concentrations were unaffected by substance P infusion or pravastatin treatment (table 3).

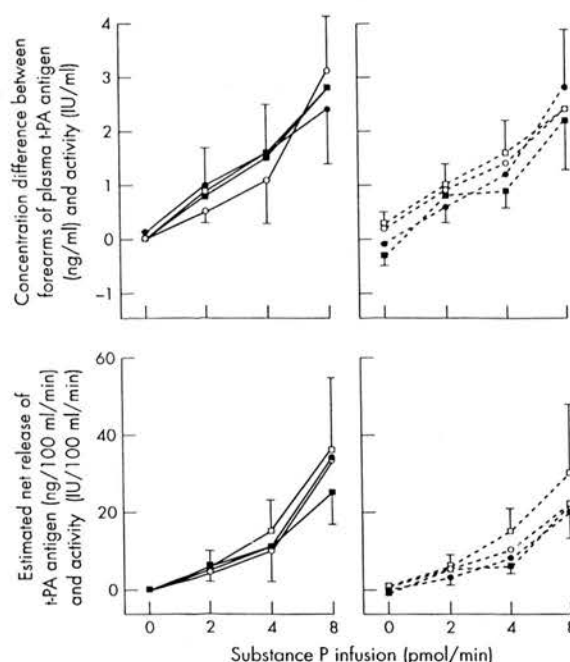
#### Reproducibility of substance P responses

Comparison of responses at baseline and during placebo treatment shows good reproducibility for substance P induced increases in plasma t-PA concentrations and FBF (table 4).

#### DISCUSSION

Despite impaired endothelium dependent forearm vasodilatation, we have shown that in patients with hypercholesterolaemia intrabrachial substance P infusions are associated with a normal capacity to release t-PA acutely. Moreover, pravastatin treatment, sufficient to reduce cholesterol concentrations by 22%, had no significant effects on acute t-PA release. This suggests that, despite the presence of endothelial dysfunction, hypercholesterolaemia does not influence the acute fibrinolytic capacity of the endothelium and that the preventative benefits of lipid lowering treatment are unlikely to be mediated by improvements in endogenous fibrinolysis.

Consistent with previous findings,<sup>1</sup> we have shown that in patients with hypercholesterolaemia, endothelium dependent vasodilatation in response to substance P infusion is impaired. However, in contrast to the pronounced impairment of t-PA



**Figure 2** Concentration difference between infused and non-infused forearms (upper panels) and estimated net release (lower panels) of plasma tissue plasminogen activator antigen (solid lines, left panels) and activity (dashed lines, right panels) during substance P administration in controls (open circles) and patients at baseline (closed circles), and during placebo (open squares) and pravastatin (closed squares) treatment.  $p < 0.001$  for each response (one way analysis of variance).

release that we have recently described in cigarette smokers,<sup>19</sup> hypercholesterolaemia and lipid lowering treatment do not appear to influence substance P induced t-PA release. These findings suggest that although smoking is associated with impaired endogenous fibrinolysis, hypercholesterolaemia is not. This is consistent with the observations that the patency rate of the infarct related artery following thrombolytic treatment during myocardial infarction is enhanced in cigarette

**Table 3** Plasma plasminogen activator inhibitor type 1 (PAI-1) antigen and activity in patients at baseline and during placebo and pravastatin treatment and in matched controls

		Patients			
Substance P dose (pmol/min)		Controls	Baseline	Placebo	Pravastatin
PAI-1 antigen (ng/ml)					
Infused arm	0	23 (9)	40 (10)	47 (12)	48 (15)
	2	24 (8)	43 (10)	47 (11)	44 (13)
	4	24 (8)	41 (9)	46 (11)	51 (16)
	8	29 (10)	39 (9)	46 (12)	46 (14)
Non-infused arm	0	22 (8)	44 (11)	50 (13)	50 (16)
	2	23 (8)	40 (9)	51 (12)	51 (18)
	4	26 (9)	39 (9)	51 (14)	46 (14)
	8	28 (8)	38 (9)	48 (11)	46 (14)
PAI-1 activity (AU/ml)					
Infused arm	0	9 (3)	13 (2)	15 (3)	13 (3)
	2	10 (4)	10 (2)	16 (3)	13 (2)
	4	9 (4)	9 (3)	13 (3)	14 (2)
	8	11 (5)	11 (3)	13 (3)	13 (2)
Non-infused arm	0	12 (3)	14 (3)	14 (3)	15 (3)
	2	8 (4)	13 (2)	16 (3)	13 (2)
	4	11 (5)	10 (3)	14 (2)	13 (3)
	8	9 (4)	12 (3)	13 (3)	12 (3)

Data are mean (SEM).

**Table 4** Repeatability of t-PA and blood flow responses to substance P administration in the infused forearm: baseline versus placebo

	Substance P dose (pmol/min)	Baseline mean	Placebo mean	Mean of differences	Coefficient of repeatability
t-PA antigen (ng/ml)					
	0	4.6 (0.8)	4.7 (0.6)	0.5	0.4
	2	5.4 (0.8)	5.4 (0.6)	0.1	1.4
	4	5.7 (0.9)	6.0 (0.9)	0.6	1.4
	8	7.4 (1.1)	7.5 (1.3)	0.1	1.6
t-PA activity (IU/ml)					
	0	0.5 (0.1)	0.6 (0.1)	0.0	0.2
	2	1.3 (0.5)	1.1 (0.4)	0.3	0.6
	4	2.0 (0.6)	1.7 (0.9)	0.4	1.2
	8	3.4 (1.1)	3.9 (1.4)	0.3	1.4
Absolute forearm blood flow (ml/100 ml/min)					
	0	3.5 (0.3)	3.2 (0.3)	0.3	0.6
	2	11.2 (0.7)	10.3 (1.3)	0.9	3.0
	4	14.4 (1.8)	12.6 (1.5)	1.7	4.6
	8	15.7 (1.1)	14.9 (1.5)	0.8	3.4
Change in forearm blood flow (%)					
	2	206 (20)	240 (30)	35	48
	4	300 (39)	300 (36)	0	68
	8	402 (49)	384 (58)	17	76

Data are mean (SEM).

smokers,<sup>10-12</sup> but not in patients with hypercholesterolaemia,<sup>11</sup> and that cigarette smoking is associated with thrombotic occlusion whereas hypercholesterolaemia is linked to atherogenesis and plaque rupture.<sup>4</sup> This would also indicate that endothelial dysfunction can be manifest in separate distinct pathways depending on the nature of the insult.

The WOSCOPS (west of Scotland coronary prevention study)<sup>14</sup> was the first major randomised controlled trial to show the primary preventative benefits of lipid lowering therapy. We therefore chose to examine the effects of pravastatin 40 mg daily on endothelial and fibrinolytic function in a well defined and otherwise healthy hypercholesterolaemic population. Although the total cholesterol was reduced by 22% and pravastatin was used at doses that have been shown to confer major preventative benefits in several large scale clinical trials,<sup>15-16</sup> the total serum cholesterol concentration remained significantly higher than that in the normocholesterolaemic population. It may be that a greater reduction in cholesterol concentrations would have facilitated a significant improvement in endothelium dependent vasodilatation. However, the mean cholesterol concentrations of the patients in

WOSCOPS were similar to those in the present study and the relative risk reduction in ischaemic events is the same across a broad range of cholesterol concentrations.<sup>15</sup> Moreover, it appears that statins do not just lower cholesterol and may have many ancillary vascular actions.<sup>17</sup> Finally, since we did not observe a significant difference in t-PA release between hypercholesterolaemic and normocholesterolaemic subjects, it is unlikely that additional reductions in lipid concentrations, brought about by higher doses of pravastatin or more potent statins, would influence the acute release of t-PA.

The influence of lipid lowering therapy on endothelial dysfunction was initially studied in patients with hypercholesterolaemia following treatment for 3-6 months.<sup>3,6</sup> However, more recent studies have shown that endothelial dysfunction can be reversed by statin treatment for six<sup>18</sup> or even four weeks.<sup>7</sup> Indeed, two hour treatment with plasma LDL apheresis is associated with rapid and immediate reversal of endothelial dysfunction.<sup>19</sup> It is therefore unlikely that the absence of an effect of pravastatin treatment is caused by the length of treatment and is consistent with a recent preliminary report from the WOSCOPS that showed no



changes in basal plasma t-PA concentrations despite 12 months' treatment with pravastatin.<sup>10</sup> Moreover, in contrast to previous studies, we have used a randomised double blind crossover study design that provides a greater sensitivity to detect potential differences in responses. The coefficients of reproducibility are consistent with our previous studies<sup>20-21</sup> and indicate a power sufficient to detect an approximately 20% change in blood flow and fibrinolytic responses.

In summary, whereas endothelium dependent vasodilatation is abnormal in both cigarette smoking and hypercholesterolaemia, acute t-PA release appears to be impaired only by cigarette smoking.<sup>19</sup> These observations support the concept that endothelial dysfunction is not a homogeneous condition and may be manifested in differing ways depending on the nature of the injury. Moreover, the benefits of lipid lowering treatment in the primary<sup>14</sup> and secondary<sup>15-16</sup> prevention of coronary events are unlikely to be mediated by improvements in endogenous fibrinolysis.

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## **PUBLICATION 15**

## Diesel Exhaust Inhalation Causes Vascular Dysfunction and Impaired Endogenous Fibrinolysis

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**Background** Although the mechanisms are unknown, it has been suggested that transient exposure to traffic derived air pollution may be a trigger for acute myocardial infarction. The study aim was to investigate the effects of diesel exhaust inhalation on vascular and endothelial function in humans.

**Methods and Results** In a double blind, randomized, cross over study, 30 healthy men were exposed to diluted diesel exhaust (300  $\mu\text{g}/\text{m}^3$  particulate concentration) or air for 1 hour during intermittent exercise. Bilateral forearm blood flow and inflammatory factors were measured before and during unilateral intrabrachial bradykinin (100 to 1000 pmol/min), acetylcholine (5 to 20  $\mu\text{g}/\text{min}$ ), sodium nitroprusside (2 to 8  $\mu\text{g}/\text{min}$ ), and verapamil (10 to 100  $\mu\text{g}/\text{min}$ ) infusions 2 and 6 hours after exposure. There were no differences in resting forearm blood flow or inflammatory markers after exposure to diesel exhaust or air. Although there was a dose dependent increase in blood flow with each vasodilator ( $P < 0.0001$  for all), this response was attenuated with bradykinin ( $P < 0.05$ ), acetylcholine ( $P < 0.05$ ), and sodium nitroprusside ( $P < 0.001$ ) infusions 2 hours after exposure to diesel exhaust, which persisted at 6 hours. Bradykinin caused a dose-dependent increase in plasma tissue plasminogen activator ( $P < 0.0001$ ) that was suppressed 6 hours after exposure to diesel ( $P < 0.001$ ; area under the curve decreased by 34%).

**Conclusions** At levels encountered in an urban environment, inhalation of dilute diesel exhaust impairs 2 important and complementary aspects of vascular function in humans: the regulation of vascular tone and endogenous fibrinolysis. These important findings provide a potential mechanism that links air pollution to the pathogenesis of atherothrombosis and acute myocardial infarction. (*Circulation*. 2005;112:3930-3936.)

**Key Words:** air pollution ■ endothelium ■ blood flow ■ fibrinolysis

Air pollution is a major cause of cardiovascular morbidity and mortality. Short-term increases in air pollution exacerbate cardiorespiratory disease, leading to hospitalization for conditions including acute myocardial infarction.<sup>1</sup> Long-term repeated exposure increases the risk of cardiovascular mortality, with deaths attributable to ischemic heart disease, arrhythmias, and heart failure.<sup>2</sup> These associations are strongest for fine particulate air pollutants ( $\text{PM}_{2.5}$ ),<sup>3</sup> of which the combustion-derived nanoparticulates of diesel exhaust are an important component.<sup>4</sup> Although significant improvements in air quality have occurred during the last 50 years, the association between  $\text{PM}_{2.5}$  and mortality is evident below current air quality standards.<sup>5</sup>

### Clinical Perspective p 3936

Despite the strength of the epidemiological evidence and the emergence of promising hypotheses,<sup>6,7</sup> the important

constituents and biological mechanisms responsible for the cardiovascular effects of air pollution are largely unknown. It was recently reported that transient exposure to road traffic may increase the risk of acute myocardial infarction.<sup>8</sup> Long-term exposure to traffic in those living within 100 m of a major road significantly increased cardiopulmonary mortality.<sup>9</sup> These important observations suggest that the combustion-derived particulates in  $\text{PM}_{2.5}$  may be critical in determining the cardiovascular effects of air pollution.

Abnormal endothelial function has been widely recognized in patients with atherosclerosis and its risk factors.<sup>10,11</sup> Endothelial dysfunction can also predict the likelihood of future cardiovascular events and death in patients with coronary artery disease<sup>12</sup> and in at risk individuals with normal coronary arteries.<sup>13</sup> We have previously demonstrated endothelial dysfunction in both the peripheral and coronary circulations

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of cigarette smokers.<sup>10,11</sup> Given the potential for common etiologic factors contained within polluted air and cigarette smoke, we hypothesized that the adverse cardiovascular effects of air pollution are a result of combustion-derived particulates and are mediated by an impairment of normal vascular function. Using a carefully characterized exposure system, we sought to assess the effect of diluted diesel exhaust inhalation on endothelial vasomotor and fibrinolytic function in humans.

## Methods

### Subjects

Thirty healthy, male nonsmokers between 20 and 38 years old participated in these studies, which were performed with the approval of the local research ethics committee, in accordance with the Declaration of Helsinki, and the written, informed consent of all volunteers. Subjects taking regular medication and those with clinical evidence of atherosclerotic vascular disease, arrhythmias, diabetes mellitus, hypertension, renal or hepatic failure, asthma, significant occupational exposure to air pollution, or an intercurrent illness likely to be associated with inflammation were excluded from the study. Subjects had normal lung function and reported no symptoms of respiratory tract infection for at least 6 weeks before or during the study.

### Study Design

Subjects attended the experimental sessions on 2 occasions 2 weeks apart and received either filtered air or diesel exhaust in a randomized, double-blind, cross-over design. Each subject was exposed for 1 hour in a specially built diesel exposure chamber according to a previously described standard protocol.<sup>14</sup> During each exposure, they performed moderate exercise (minute ventilation, 25 L · min<sup>-1</sup> · m<sup>-2</sup>) on a bicycle ergometer that was alternated with rest at 15-minute intervals.

Based on previous exposure<sup>15</sup> and systemic inflammatory<sup>16</sup> studies, vascular assessments were performed in 15 subjects at 6 to 8 hours after diesel or air exposure. In light of our findings from this 6- to 8-hour study, we subsequently determined vascular function in another 15 subjects at an earlier time point of 2 to 4 hours after exposure to diesel exhaust or air. All subjects abstained from alcohol for 24 hours and from food, tobacco, and caffeine-containing drinks for at least 4 hours before each vascular study. Studies were carried out in a quiet, temperature-controlled room maintained at 22°C to 24°C with subjects lying supine. All subjects remained indoors between the exposure and vascular assessment to minimize additional exposure to particulate air pollution.

### Diesel Exposure

The diesel exhaust was generated from an idling Volvo diesel engine (Volvo TD45, 4.5 L, 4 cylinders, 680 rpm) as described previously.<sup>15</sup> More than 90% of the exhaust was shunted away, and the remaining part was diluted with air and fed into the exposure chamber at a steady-state concentration. The air in the exposure chamber was continuously monitored for nitrogen oxides (NO, NO<sub>2</sub>), carbon monoxide (CO), particulates (number/cm<sup>3</sup>), and total hydrocarbons. The exposures were standardized by keeping the particulate concentration at 300 µg/m<sup>3</sup> and were associated with concentrations of NO<sub>2</sub> of 1.6 ppm; of NO, 4.5 ppm; of CO, 7.5 ppm; of total hydrocarbons, 4.3 ppm; of formaldehyde, 0.26 µg/m<sup>3</sup>; and of suspended particles, 1.2 × 10<sup>6</sup>/cm<sup>3</sup>. The temperature and humidity in the chamber were controlled at 20°C and 50%, respectively.

### Vascular Studies

All subjects underwent brachial artery cannulation with a 27-standard wire gauge steel needle under controlled conditions. After a 30-minute baseline saline infusion, acetylcholine at 5, 10, and 20 µg/min (endothelium-dependent vasodilator that does not release

tissue plasminogen activator [t-PA]; Merck Biosciences); bradykinin at 100, 300, and 1000 pmol/min (endothelium-dependent vasodilator that releases t-PA; Merck Biosciences); and sodium nitroprusside at 2, 4, and 8 µg/min (endothelium-independent vasodilator that does not release t-PA; David Bull Laboratories) were infused for 6 minutes at each dose. The 3 vasodilators were separated by 20-minute saline infusions and given in a randomized order. In the second cohort with the early (2- to 4-hour) vascular assessment, verapamil at 10, 30, and 100 µg/min (endothelium- and NO-independent vasodilator that does not release t-PA) was infused at the end of the study protocol.<sup>17</sup>

Forearm blood flow (FBF) was measured in infused and noninfused arms by venous occlusion plethysmography with a mercury-in-silicone elastomer strain gauges as described previously.<sup>18</sup> Supine heart rate and blood pressure in the noninfused arm were monitored at intervals throughout each study with a semiautomated, noninvasive, oscillometric sphygmomanometer.

Venous cannulas (17 gauge) were inserted into large subcutaneous veins of the antecubital fossae of both arms. Blood (10 mL) was withdrawn simultaneously from each arm at baseline and during infusion of each dose of bradykinin and collected into acidified buffered citrate (Stabilyte tubes, Biopool International) for t-PA assays and into citrate (BD Vacutainer) for plasminogen activator inhibitor type 1 (PAI-1) assays. Samples were kept on ice before being centrifuged at 2000g for 30 minutes at 4°C. Platelet-free plasma was decanted and stored at -80°C before assay. Plasma t-PA and PAI-1 antigen concentrations were determined by ELISAs (TintElize t-PA, Biopool EIA; Coaliza PAI-1, Chromogenix AB). Hematocrit was determined by capillary tube centrifugation at baseline and during infusion of bradykinin at 1000 pmol/min.

Blood samples were taken immediately before and at 2 and 6 hours after exposure and analyzed for total cells, differential cell counts, and platelets by an autoanalyzer. Plasma interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) were measured with commercially available ELISAs (Quantikine, R&D Systems). Plasma immunoreactive big endothelin (ET)-1 and ET-1 concentrations were measured according to an acetic acid extraction technique by use of a modified commercial radioimmunoassay with rabbit anti-human big ET-1 or ET-1 (Peninsula Laboratories Europe), as described previously.<sup>19</sup> Serum C-reactive protein (CRP) concentrations were measured with an immunonephelometric assay (Behring BN II nephelometer).

### Data Analysis and Statistics

Plethysmographic data were analyzed as described previously.<sup>10</sup> The estimated net release of t-PA antigen was defined as the product of the infused forearm plasma flow (based on the mean hematocrit and the infused FBF) and the concentration difference between the infused and noninfused arms.<sup>10</sup> Continuous variables are reported as mean ± SEM. Statistical analyses were performed with GraphPad Prism (Graph Pad Software) by ANOVA with repeated measures and 2-tailed Student *t* test, where appropriate. The area under the curve was calculated for the estimated net release of t-PA during the forearm study period. Statistical significance was taken at *P* < 0.05.

## Results

There were no differences in resting heart rate, blood pressure, or baseline FBF after exposure to diesel exhaust or air in either cohort (Table 1). Leukocyte, neutrophil, and platelet counts; plasma IL-6, TNF-α, big ET-1, and ET-1; and serum CRP concentrations were not altered by diesel or air exposure (Table 2).

Bradykinin, acetylcholine, and sodium nitroprusside caused dose-dependent increases in FBF after both air and diesel exhaust exposure (*P* < 0.0001; Figure 1). The increase in blood flow was blunted 2 hours after exposure to diesel exhaust in response to infusion of bradykinin (*P* < 0.05), acetylcholine (*P* < 0.05), and sodium nitroprusside (*P* < 0.001), and this dimin-

TABLE 1. Baseline Hemodynamics Variables

	Air	Diesel	Significance
2 Hours, n=15			
Heart rate, bpm	64±3	65±2	P=0.64
Systolic blood pressure, mm Hg	140±4	148±4	P=0.13
Diastolic blood pressure, mm Hg	71±3	77±4	P=0.08
Infused FBF, mL/100 mL tissue per min	3.3±0.6	3.1±0.4	P=0.45
Noninfused FBF, mL/100 mL tissue per min	2.3±0.2	2.6±0.4	P=0.30
6 Hours, n=15			
Heart rate, bpm	61±2	60±2	P=0.66
Systolic blood pressure, mm Hg	138±5	138±3	P=0.39
Diastolic blood pressure, mm Hg	75±2	76±4	P=0.87
Infused FBF, mL/100 mL tissue per min	3.1±0.5	2.5±0.2	P=0.25
Noninfused FBF, mL/100 mL tissue per min	2.2±0.1	2.4±0.3	P=0.65

Values are reported as mean±SEM, 2-tailed paired *t* test.

ished response persisted at 6 hours (Figure 2). In contrast, verapamil induced vasodilatation was unaffected after exposure to air or diesel exhaust (*P*=NS).

Bradykinin caused a dose-dependent increase in plasma t-PA antigen concentrations (*P*<0.0001; Table 3) that was reduced 6 hours after diesel exposure (*P*<0.001). The estimated net t-PA antigen release was reduced by 34% 6 hours after exposure to diesel (*P*<0.05; Figure 3) but was unaffected at the earlier time point of 2 hours.

TABLE 2. Systemic Effects of Exposure to Diesel Exhaust

	Before Exposure	2 Hours	6 Hours
Air			
Leucocytes, ×10 <sup>9</sup> cells/L	5.1±0.2	5.6±0.3	5.3±0.3
Neutrophils, ×10 <sup>9</sup> cells/L	2.8±0.2	3.3±0.2	3.0±0.2
Platelets, ×10 <sup>9</sup> cells/L	217±12	216±9	218±12
IL-6, pg/mL	2.6±1.3	...	3.4±0.8
TNF-α, pg/mL	16.9±1.1	...	17.8±1.2
CRP, mg/L	0.9±0.3	...	0.8±0.2
PAI-1 antigen, ng/mL	20.2±3.9	19.2±2.8	18.5±3.7
t-PA antigen, ng/mL	6.6±0.6	7.0±0.6	6.0±0.5
ET-1, pg/mL	4.9±0.5	4.9±0.5	4.9±0.4
Big ET-1, pg/mL	28.8±1.5	29.6±2.5	32.2±2.5
Diesel			
Leucocytes, ×10 <sup>9</sup> cells/L	5.6±0.3	5.7±0.4	5.1±0.3
Neutrophils, ×10 <sup>9</sup> cells/L	2.8±0.2	3.4±0.3	2.9±0.3
Platelets, ×10 <sup>9</sup> cells/L	228±14	227±11	221±12
IL-6, pg/mL	2.7±0.7	...	4.3±2.2
TNF-α, pg/mL	15.3±0.5	...	16.0±0.8
CRP, mg/L	1.0±0.4	...	0.7±0.4
PAI-1 antigen, ng/mL	16.1±3.0	15.7±3.6	12.8±2.6
t-PA antigen, ng/mL	6.0±0.6	5.9±0.6	5.3±0.6
ET-1, pg/mL	4.5±0.4	4.8±0.3	4.8±0.5
Big ET-1, pg/mL	30.2±2.3	31.9±3.7	28.1±2.6

Values are reported as mean±SEM.

## Discussion

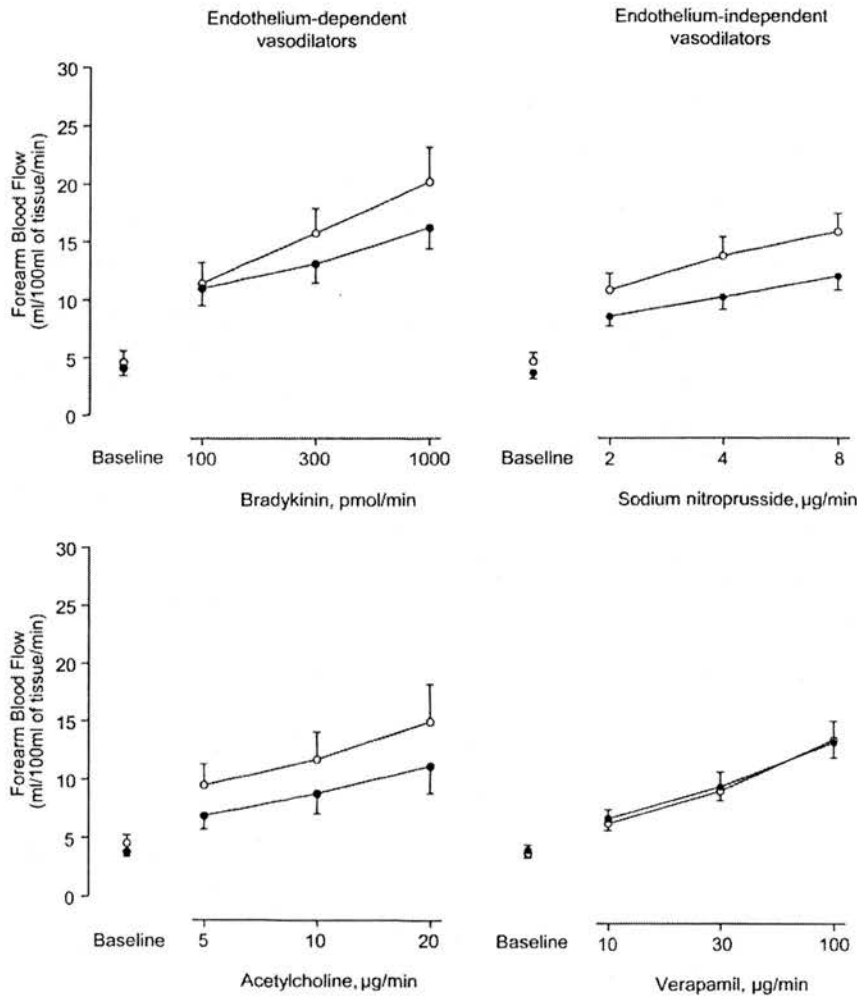
This is the first study to demonstrate that inhalation of diesel exhaust, a common urban air pollutant, can impair vascular function in humans. Using a robust and powerful study design, we have assessed 2 important and complementary aspects of vascular function: the regulation of vascular tone and endogenous fibrinolysis. Both are impaired and plausibly related to the well-documented cardiovascular effects of air pollution. These important findings provide a plausible mechanism that links air pollution to the pathogenesis of atherosclerosis and acute myocardial infarction.

## Vasomotor Function

Impaired endothelium-dependent and -independent vasomotor function in the forearm vascular bed is associated with an increased risk of acute cardiovascular events, including cardiac death.<sup>12</sup> We have demonstrated that inhalation of diesel exhaust impairs vasomotor responses to both endothelium-dependent and -independent vasodilators at 6 hours. On the basis of this initial study, it is unclear whether the impairment is primarily mediated by the vascular endothelium or is a result of smooth muscle dysfunction. However, reduced NO bioavailability in the presence of increased systemic or vascular oxidative stress is an attractive hypothesis.

The endothelium is a major target of oxidative stress, and this interaction plays an important role in the pathophysiology of vascular disease.<sup>20</sup> Superoxide radicals, produced as a consequence of oxidative stress, combine with NO to form peroxynitrite, thus reducing NO bioavailability in the vessel wall and shifting the balance toward vasoconstriction. In vascular smooth muscle cells, superoxide inhibits the activity of enzymes such as soluble guanylyl cyclase<sup>21</sup> and cGMP-dependent protein kinase,<sup>22</sup> thereby reducing both endothelium-dependent and -independent NO-mediated vasodilation.

We hypothesized that our initial findings were due to the oxidative effects of diesel exhaust, and as such, vascular impairment would occur early. In the subsequent study, we have demonstrated an acute impairment to endothelium-dependent and -independent vasodilators, but we were also



**Figure 1.** Infused FBF in subjects 2 to 4 hours after diesel exposure (●) and air (○) during intrabrachial infusion of bradykinin, acetylcholine, sodium nitroprusside, and verapamil. For all dose responses,  $P < 0.0001$ . For diesel exposure (●) vs air (○), bradykinin  $P < 0.05$ , acetylcholine  $P < 0.05$ , sodium nitroprusside  $P < 0.001$ , and verapamil  $P = \text{NS}$ .

able to show that vasodilation to the calcium channel antagonist verapamil was unaffected. This suggests that the mechanism of vascular dysfunction involves increased consumption of NO, whether it be endogenously derived from endothelial NO synthase or from an exogenous source, such as sodium nitroprusside. Indeed, *in vitro* studies provide support for this mechanism, with Ikeda et al<sup>23</sup> demonstrating that incubation of aortic ring preparations with diesel exhaust particles resulted in a dose-dependent inhibition of acetylcholine-mediated relaxation, an effect abolished by incubation with superoxide dismutase.

Our findings of an acute effect of exposure to air pollution are consistent with recent epidemiological studies that report a significant increase in risk of acute myocardial infarction as little as 2 hours after exposure to road traffic<sup>8</sup> or an increase in  $\text{PM}_{2.5}$ .<sup>1</sup> Our studies add to those of Brook et al,<sup>24</sup> who demonstrated a reduction in brachial artery diameter immediately after exposure to a mixture of concentrated ambient particles and ozone. In contrast, they did not find an effect on endothelium-dependent or -independent vasodilation by flow-mediated and nitroglycerine-induced dilation. This may reflect differences in the potency of the pollution models used or the technique used to assess vascular function. Exposures to concentrated ambient particulates are inherently variable in

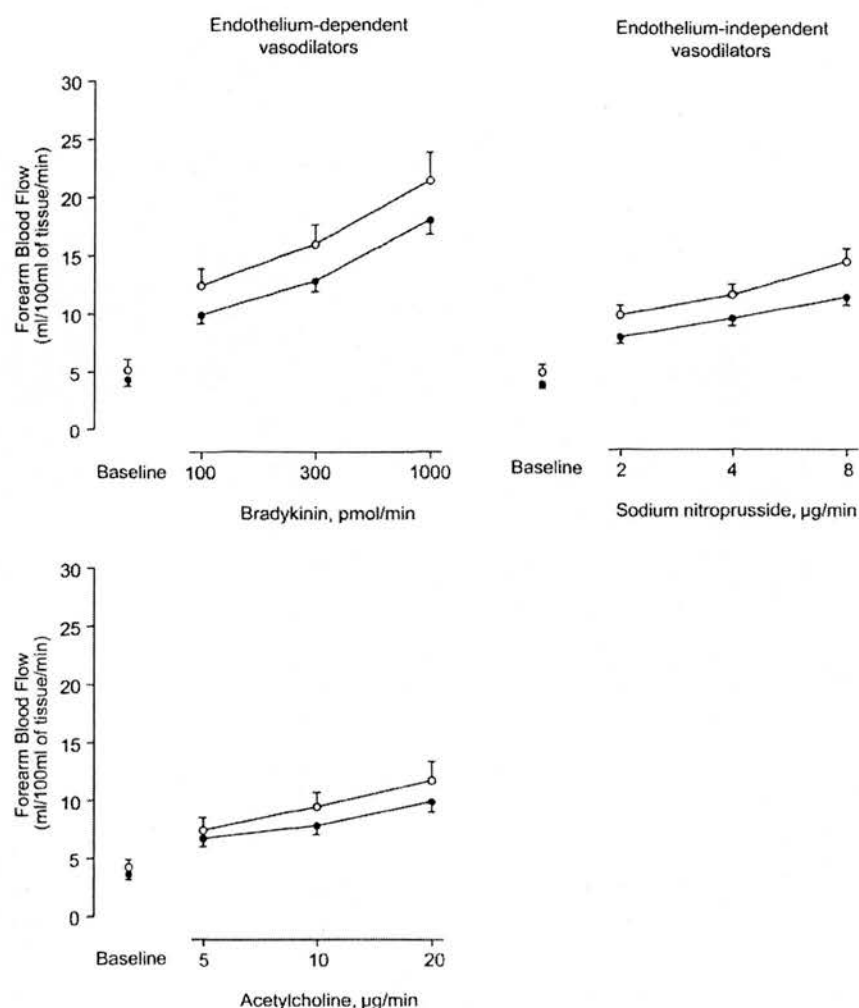
magnitude and composition, whereas in our study, each volunteer received a standard exposure to combustion-derived particulates of known toxicity. Alternatively, it is possible that the vascular effects of particulate matter are mediated primarily in the resistance vessels, as assessed by plethysmography, rather than in the conduit arteries, as assessed by ultrasound of the brachial artery.

### Fibrinolytic Function

Acute endogenous t-PA release from the endothelium regulates the dissolution of intravascular thrombus and is a critical determinant of cardiovascular outcome. This is exemplified by the clinical observation that in  $\approx 30\%$  of patients with acute myocardial infarction, spontaneous reperfusion occurs within 12 hours of vessel occlusion. The increased risk of atherothrombosis and myocardial infarction in cigarette smokers is at least in part explained by impaired fibrinolytic capacity.<sup>10,11</sup>

We have described an impairment in acute endogenous fibrinolytic capacity after diesel exhaust inhalation. This abnormality may have prothrombotic consequences that could plausibly result in acute cardiovascular events.<sup>8</sup> t-PA release was reduced 6 hours after exposure but not at the earlier time point, suggesting that this impairment is mediated by an inducible pathway or a change in protein synthesis.





**Figure 2.** Infused FBF in subjects 6 to 8 hours after diesel exposure (●) and air (○) during intrabrachial infusion of bradykinin, acetylcholine, and sodium nitroprusside. For all dose responses,  $P<0.0001$ . For diesel exposure (●) vs air (○), bradykinin  $P<0.05$ , acetylcholine  $P=0.07$ , and sodium nitroprusside  $P<0.001$ .

Indeed, culture of human umbilical vein endothelial cells with particulate matter for 6 hours inhibits both the synthesis and release of t-PA in a dose-dependent manner.<sup>25</sup> Given that cigarette smoking and air pollution share common toxicolog-

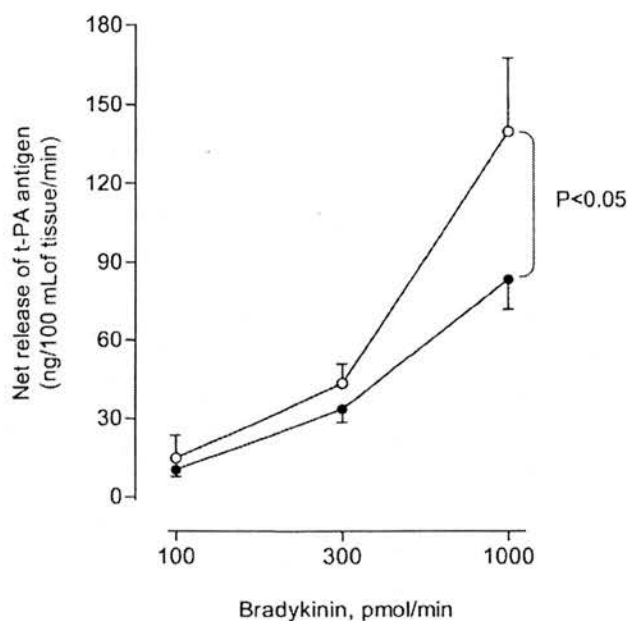
ical properties, the present findings are consistent with previous observations in the peripheral<sup>10</sup> and coronary<sup>11</sup> circulations of cigarette smokers and suggest a potential common etiologic factor.

**TABLE 3. Plasma t-PA Antigen Concentrations After Air and Diesel Exposure**

	Air (Bradykinin, pmol/min)				Diesel (Bradykinin, pmol/min)			
	0	100	300	1000	0	100	300	1000
<b>2 Hours</b>								
tPA antigen, ng/mL								
Noninfused arm	7.0±0.6	7.0±0.7	7.7±0.8	9.4±1.2	7.2±0.8	7.5±0.8	8.0±0.8	8.9±0.8
Infused arm	6.5±0.5	8.8±1.4	12.2±1.9	17.1±1.3*	6.6±0.7	9.8±1.5	13.7±0.9	18.7±2.7*
Difference	-0.5±0.3	1.7±0.8	4.6±1.2	9.3±1.1*	-0.3±0.4	2.2±0.8	5.3±1.4	9.9±2.2*
Net t-PA release, ng/100 mL of tissue per min	-3.3±2.2	6.6±2.7	27.3±4.3	81.3±10.7*	-0.2±1.0	8.9±3.0	29.4±6.1	82.9±17.8*
<b>6 Hours</b>								
tPA antigen, ng/mL								
Noninfused arm	5.8±0.5	7.1±1.1	7.0±0.6	7.7±0.6	5.5±0.6	5.6±0.5	5.7±0.4	7.1±0.5
Infused arm	6.0±0.5	9.6±1.7	12.0±1.3	18.6±2.5*	5.3±0.6	7.4±0.9	10.0±1.0	14.5±1.3*†
Difference	0.2±0.2	2.6±1.7	5.4±1.1	11.7±2.5*	-0.2±0.2	2.0±0.6	4.6±0.8	8.0±1.1*†
Net t-PA release, ng/100 mL of tissue per min	0.7±0.9	15.8±9.4	42.9±7.3	138.2±27.8*	-1.0±0.5	10.6±2.9	33.2±5.1	82.5±11.5*†

Values are reported as mean±SEM.

ANOVA (dose response), \* $P<0.0001$ ; ANOVA (air vs diesel), † $P<0.05$ , ‡ $P<0.001$ .



**Figure 3.** Net release of t-PA antigen in subjects 6 hours after diesel exposure (●) and air (○) during intrabrachial infusion of bradykinin. For both dose responses,  $P < 0.0001$ . For diesel exposure (●) vs air (○),  $P < 0.05$ .

### Air Pollution, Oxidative Stress, and Inflammation

A substantial body of evidence supports a role for oxidative stress in determining the toxicity of ambient pollution<sup>26</sup> and in the proinflammatory effects of diesel exhaust particles.<sup>27,28</sup> Reactive oxidant species arise not only from the redox potential of the pollutants themselves but also from the activation of alveolar epithelial cells or resident macrophage and the recruitment of circulating neutrophils.

The potential for inhaled nanoparticulate air pollution to cause local inflammation is not in doubt, and airway neutrophilia has been demonstrated in a healthy volunteer study with the same concentration of diesel particulate and exposure system.<sup>15</sup> In our study, inhaled diesel exhaust was not associated with an increase in blood leukocytes, plasma IL-6 and TNF- $\alpha$ , or serum CRP concentrations, but this does not rule out the influence of other circulating inflammatory factors, oxidized lipids, or proteins.

### Population Risk and Exposure

As an important source of combustion-derived particulate, diesel exhaust is strongly implicated in the observed adverse effects of air pollution.<sup>4,29,30</sup> Particulate matter concentrations can regularly reach levels of 300  $\mu\text{g}/\text{m}^3$  in heavy traffic, occupational settings, and the world's largest cities.<sup>31</sup> Exposure to 300  $\mu\text{g}/\text{m}^3$  for 1 hour increases a person's average exposure during a 24-hour period by only 12  $\mu\text{g}/\text{m}^3$ . Changes of this magnitude occur on a daily basis in even the least polluted of cities and are associated with increases in cardiorespiratory mortality.<sup>3</sup> Our model is therefore relevant in both the composition and magnitude of exposure for the assessment of short-term health effects in humans.

Diesel exhaust is a complex mixture of gases and particles, and from our findings, we cannot exclude a nonparticulate

cause of the adverse vascular effects. However, in epidemiological studies, particulate matter has been held responsible for the majority of the adverse health effects of air pollution.<sup>32</sup> Ambient NO<sub>2</sub> can be considered a surrogate for traffic-derived pollution,<sup>29</sup> but it has little adverse effect in controlled chamber studies, even at the exposure levels seen here.<sup>33</sup> There are no reports of the potential adverse cardiovascular effects of toxins such as hydrocarbons or formaldehyde. We therefore suggest that the vascular effects described herein are mediated primarily by diesel exhaust particulates and not its other components, but this needs to be more definitively addressed.

### Conclusions

Exposure to increased levels of combustion-derived air pollution for as little as 1 hour can impair vasomotor function and endogenous fibrinolysis in humans. We provide evidence that this may be the result of reduced NO bioavailability in the vasculature and postulate that this effect is mediated by oxidative stress induced by the nanoparticulate fraction of diesel exhaust. These data provide a plausible mechanistic link to explain the association between air pollution and acute myocardial infarction.

### Acknowledgments

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### Disclosures

None.

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### CLINICAL PERSPECTIVE

Air pollution is a serious problem in the world's major cities owing to the combustion of fossil fuels such as diesel oil. In particular, there has been recent interest in the consistent association between increased levels of air pollution and cardiovascular morbidity and mortality. The World Health Organization estimates that a quarter of the world's population is exposed to unhealthy concentrations of air pollutants. The American Heart Association recently issued a scientific statement highlighting the increased cardiovascular risk associated with exposure to air pollution and emphasized the importance of establishing a mechanistic link to explain these epidemiological observations. We have previously demonstrated vascular dysfunction in cigarette smokers. Because combustion products and particulate matter are common to both polluted air and cigarette smoke, we hypothesized that air pollution would cause detrimental vascular effects. This is the first study to demonstrate that inhalation of diesel exhaust, a common urban air pollutant, can impair vascular function in humans. Using a double-blind, randomized, cross-over study design, we have assessed the effects of diesel exhaust inhalation on 2 important and complementary aspects of vascular function: the regulation of vascular tone and endogenous fibrinolysis. We were able to demonstrate that both are impaired and plausibly related to the well-documented adverse cardiovascular effects of air pollution. These important findings provide a plausible mechanism that links air pollution to the pathogenesis of atherothrombosis and acute myocardial infarction.

**PUBLICATION 16**

# Persistent Endothelial Dysfunction in Humans after Diesel Exhaust Inhalation

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**Rationale:** Exposure to combustion-derived air pollution is associated with an early (1–2 h) and sustained (24 h) rise in cardiovascular morbidity and mortality. We have previously demonstrated that inhalation of diesel exhaust causes an immediate (within 2 h) impairment of vascular and endothelial function in humans.

**Objectives:** To investigate the vascular and systemic effects of diesel exhaust in humans 24 hours after inhalation.

**Methods:** Fifteen healthy men were exposed to diesel exhaust (particulate concentration, 300 µg/m<sup>3</sup>) or filtered air for 1 hour in a double-blind, randomized, crossover study. Twenty-four hours after exposure, bilateral forearm blood flow, and inflammatory and fibrinolytic markers were measured before and during unilateral intrabrachial bradykinin (100–1,000 pmol/min), acetylcholine (5–20 µg/min), sodium nitroprusside (2–8 µg/min), and verapamil (10–100 µg/min) infusions.

**Measurements and Main Results:** Resting forearm blood flow, blood pressure, and basal fibrinolytic markers were similar 24 hours after either exposure. Diesel exhaust increased plasma cytokine concentrations (tumor necrosis factor-α and interleukin-6,  $p < 0.05$  for both) but appeared to reduce acetylcholine ( $p = 0.01$ ), and bradykinin ( $p = 0.08$ ) induced forearm vasodilatation. In contrast, there were no differences in either endothelium-independent (sodium nitroprusside and verapamil) vasodilatation or bradykinin-induced acute plasma tissue plasminogen activator release.

**Conclusions:** Twenty-four hours after diesel exposure, there is a selective and persistent impairment of endothelium-dependent vasodilatation that occurs in the presence of mild systemic inflammation. These findings suggest that combustion-derived air pollution may have important systemic and adverse vascular effects for at least 24 hours after exposure.

**Keywords:** air pollution; endothelium; blood flow; inflammation; diesel exhaust

The link between ambient particulate matter (PM), air pollution, and increased cardiorespiratory mortality and morbidity is well

## AT A GLANCE COMMENTARY

### Scientific Knowledge on the Subject

The link between ambient particulate matter air pollution and cardiorespiratory mortality and morbidity is well established. However, the biological mechanisms underlying the cardiovascular effects of particulate matter air pollution are largely unknown.

### What This Study Adds to the Field

Exposure to diesel exhaust causes a selective impairment of vascular endothelial function, which persists up to 24 hours after exposure. Adverse cardiovascular effects of combustion-derived pollution may be mediated through prolonged detrimental vascular effects.

established (1). Short-term exposure to traffic and ambient air pollution is associated with an increased risk of early (1–2 h) and delayed (24 h) presentation with acute myocardial infarction (2, 3), or rehospitalization for myocardial ischemia in patients with prior myocardial infarction (4). Long-term repeated exposure to PM pollution increases the risk of cardiovascular mortality, with deaths attributable to ischemic heart disease, arrhythmia, heart failure, and cardiac arrest (5–7). These associations are strongest for fine particulate air pollutants ( $\leq 2.5$  µm in diameter; PM<sub>2.5</sub>) (8). Diesel exhaust emissions are a significant source of PM<sub>2.5</sub> in urban environments, particularly in Europe where the use of diesel engines in transport has increased steadily in recent years (9). As a consequence, diesel exhaust exposures have been used as models of PM pollution in experimental studies (10–12).

The biological mechanisms underlying the cardiovascular effects of PM air pollution are largely unknown, although it has been suggested that pulmonary inflammation results in systemic consequences that adversely affect the cardiovascular system (13). *In vitro* studies, animal models, and human exposures have clearly established the oxidant and proinflammatory nature of combustion-derived PM and implied a role for oxidative stress in determining the toxicity of ambient air pollution and the proinflammatory effects of diesel exhaust particulates (14–16). At levels encountered in an urban environment, we have previously demonstrated that exposure to diesel exhaust causes pronounced airway inflammation, including recruitment of inflammatory cells, the up-regulation of vascular endothelial adhesion molecules, and the enhanced epithelial expression of cytokines. These effects are associated with the up-regulation of

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important oxidative stress-related transcription factors and mitogen-activated protein (MAP) kinases in the bronchial epithelium (11, 12, 17–20).

Endothelial dysfunction is widely considered to represent the earliest pathologic process in atherosclerosis (21), with established risk factors for cardiovascular disease adversely affecting endothelial function (22, 23). In recent studies, we have demonstrated an immediate impairment of vascular and endogenous fibrinolytic function in young healthy volunteers after exposure to diesel exhaust (24). In the absence of systemic inflammation up to 6 hours after exposure, we suggested that these early vascular effects were determined by oxidative stress. The duration of these adverse vascular effects are unknown, and the potential for developing later pulmonary and systemic inflammatory effects to potentiate vascular dysfunction requires further investigation.

The aim of the present study was to investigate whether there is systemic inflammation and sustained vascular dysfunction in healthy volunteers 24 hours after exposure to diesel exhaust.

## METHODS

### Study Design

Fifteen healthy male nonsmokers (mean age, 26 yr; range, 18–38 yr) participated in the study. Subjects were exposed to filtered ambient air or diesel exhaust at a particulate concentration of 300  $\mu\text{g}/\text{m}^3$ , in a randomized, double-blind, crossover fashion for 1 hour, according to a previously described standard protocol (11). During each exposure, subjects alternated moderate exercise on a bicycle ergometer and rest at 15-minute intervals. Vascular assessments were performed 24 hours after each exposure, with at least 2 weeks allowed between exposures. Subjects also underwent an identical vascular assessment 2 to 4 hours after each exposure; the results of this assessment are reported in full elsewhere (24). The study was performed in accordance with the Declaration of Helsinki, with the approval of the local ethics committee, and with the written, informed consent of each volunteer.

### Diesel Exposure

The diesel exhaust was generated from an idling Volvo diesel engine as described previously (10, 11). The air in the exposure chamber was continuously monitored for gaseous pollutants, with exposures standardized on levels of nitrogen oxides to deliver a particulate concentration of 300  $\mu\text{g}/\text{m}^3$ . The temperature and humidity in the chamber were controlled at 20°C and 50%, respectively.

### Vascular Study

The vascular studies were carried out in a quiet temperature-controlled room (22°–24°C) with subjects resting in the supine position. All subjects underwent unilateral brachial artery cannulation with a 27-standard wire gauge steel needle under controlled conditions. After a 30-minute saline infusion, acetylcholine at 5, 10, and 20  $\mu\text{g}/\text{minute}$  (endothelium-dependent vasodilator that does not release tissue plasminogen activator [t-PA]); bradykinin at 100, 300, and 1,000 pmol/minute (endothelium-dependent vasodilator that releases t-PA); and sodium nitroprusside at 2, 4, and 8  $\mu\text{g}/\text{minute}$  (endothelium-independent vasodilator that does not release t-PA) were infused for 6 minutes at each dose. The three vasodilators were separated by 20-minute saline infusions and given in a randomized order. Verapamil at 10, 30, and 100  $\mu\text{g}/\text{minute}$  (endothelium and nitric oxide-independent vasodilator that does not release t-PA) was infused for 6 minutes at each dose at the end of the study protocol (25). Forearm blood flow was measured in both arms by venous occlusion plethysmography as described previously (23). Supine heart rate and blood pressure in the noninfused arm were monitored at regular intervals.

Venous cannulae (17-gauge) were inserted into both arms. Blood was drawn simultaneously from each arm at baseline and during the infusion of each dose of bradykinin, and collected for estimation of plasma t-PA and plasminogen activator inhibitor type 1 (PAI-1) concentrations. Hematocrit was determined at baseline and after infusion of bradykinin at 1,000 pmol/minute.

## Systemic Inflammation and Oxidative Stress

Blood samples were taken before and 24 hours after the exposure and analyzed for total cells and differential and platelet counts. Plasma and serum were prepared for the measurement of cytokines (tumor necrosis factor [TNF]- $\alpha$ , IL-6), C-reactive protein, nitrite, total antioxidant capacity of plasma, soluble P-selectin, and soluble intercellular adhesion molecule 1 (sICAM-1). Diesel exhaust particles were collected on Teflon filters during exposures, and electron paramagnetic resonance (EPR) was used to establish the oxidative radical generation of particulate (see the online supplement for details).

## Data Analysis and Statistics

Plethysmographic data were analyzed as described previously (23). Estimated net release of t-PA antigen was defined as the product of the infused forearm plasma flow and the concentration difference between the infused and noninfused arms (26). Continuous variables are reported as mean  $\pm$  SEM. Statistical analyses were performed with GraphPad Prism (Graph Pad Software, Inc., San Diego, CA) using analysis of variance with repeated measures and two-tailed Student's *t* test where appropriate. Statistical significance was taken at  $p < 0.05$ .

## RESULTS

There were no differences in resting heart rate, blood pressure, or baseline forearm blood flow between or during the two study visits (Table 1).

### Vascular Function

After both air and diesel exhaust exposures, there were dose-dependent increases in the infused forearm blood flow with all vasodilators ( $p < 0.001$  for all). After exposure to diesel exhaust, endothelium-dependent vasodilation was reduced with acetylcholine ( $p = 0.01$ ) and appeared to be reduced with bradykinin ( $p = 0.08$ ; Figure 1). In contrast, there were no effects on endothelium-independent vasodilation: peak blood flow responses to sodium nitroprusside ( $14.2 \pm 1.2$  vs.  $12.8 \pm 0.8$  ml/100 ml/min) and verapamil ( $14.6 \pm 0.9$  vs.  $13.4 \pm 0.9$  ml/100 ml/min) were similar.

Venous plasma concentrations of nitrite were not effected by exposure (Table 2).

Bradykinin caused a dose-dependent increase in plasma t-PA concentrations ( $p < 0.001$ ; Table 3), which was unaffected by diesel exhaust exposure. The estimated net t-PA antigen release was similar after exposure to both diesel exhaust and filtered air (Figure 2).

All subjects also underwent an identical early vascular assessment at 2 to 4 hours after exposure, the results of which are published in full elsewhere (24). Peak forearm blood flow response to endothelium-dependent vasodilators correlates well between the early (2–4 h) and late (24–26 h) vascular assessments for bradykinin ( $r = 0.60$ ,  $p < 0.05$ ) and acetylcholine ( $r = 0.72$ ,  $p < 0.05$ ). There was also a relationship between the magnitude of impairment caused by exposure to diesel exhaust (difference between peak forearm blood flow after diesel exhaust and filtered

TABLE 1. BASELINE HEMODYNAMIC VARIABLES 24 HOURS AFTER EXPOSURE

	Air	Diesel	Significance
Heart rate, beats/min	62 $\pm$ 2	61 $\pm$ 2	$p = 0.73$
Systolic blood pressure, mm Hg	144 $\pm$ 3	143 $\pm$ 3	$p = 0.63$
Diastolic blood pressure, mm Hg	71 $\pm$ 2	75 $\pm$ 2	$p = 0.16$
Infused FBF, ml/100 ml tissue/min	3.6 $\pm$ 0.5	3.2 $\pm$ 0.4	$p = 0.15$
Noninfused FBF, ml/100 ml tissue/min	2.5 $\pm$ 0.2	2.7 $\pm$ 0.2	$p = 0.48$

Definition of abbreviation: FBF = forearm blood flow.

Values are reported as mean  $\pm$  SEM, two-tailed paired *t* test.

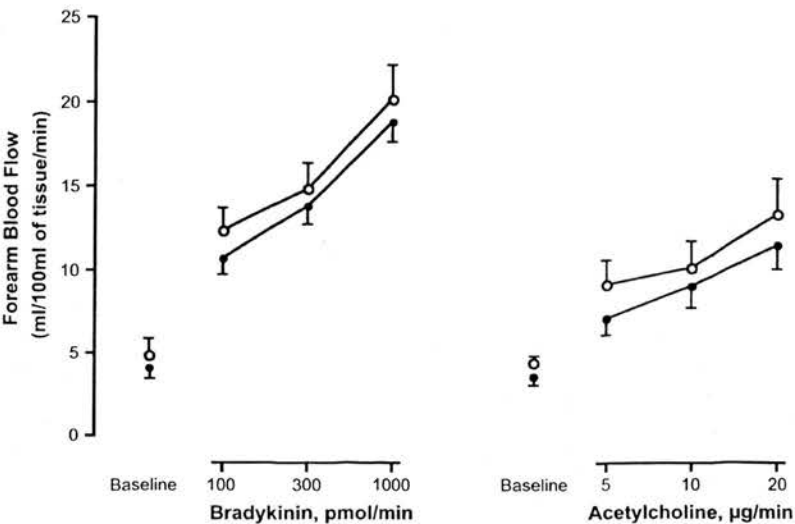


Figure 1. Infused forearm blood flow in subjects after diesel exposure (solid circles) and filtered air (open circles) during intrabrachial infusion of bradykinin and acetylcholine.  $p < 0.001$  for all dose responses in the infused arm. For diesel exposure versus air: bradykinin ( $p = 0.08$ ) and acetylcholine ( $p = 0.01$ ).

air) at the early and late time points for bradykinin ( $r = 0.58$ ,  $p < 0.05$ ), but not for the other vasodilators.

Systemic Inflammation and Oxidative Stress

Twenty-four hours after the exposures, there were no differences in leukocyte and neutrophil counts or plasma sICAM-1, t-PA, and PAI-1 antigen concentrations (Table 2). Exposure to diesel exhaust increased plasma IL-6 ( $2.2 \pm 0.2$  vs.  $1.5 \pm 0.2$  pg/ml,  $p = 0.02$ ) and TNF- $\alpha$  ( $0.99 \pm 0.07$  vs.  $0.88 \pm 0.07$  pg/ml,  $p = 0.02$ ) concentrations compared with air. Total platelet numbers were not affected by exposure, but concentrations of soluble P-selectin were increased 24 hours after exposure to diesel exhaust ( $36.5 \pm 1.4$  vs.  $33.7 \pm 1.8$  ng/ml,  $p = 0.02$ ). Total antioxidant capacity of plasma (TEAC [Trolox equivalent antioxidant capacity]) was also greater 24 hours after exposure to diesel exhaust compared with filtered air (Table 2).

The majority of inflammatory markers were significantly reduced at 24 hours when compared with baseline after exposure to both filtered air and diesel exhaust. This consistent response across exposures is due to an effect of one or more of the study interventions, which included a period of aerobic exercise, a

regulated healthy diet, a prolonged fast, and repeated blood sampling. Significant differences between exposures occurred with absolute measures at 24 hours and change from baseline in IL-6, TNF- $\alpha$ , TEAC, and soluble P-selectin concentrations were consistent and due to modification of these pathways evoked by exposure to diesel exhaust. These data are presented in full in the online supplement.

Suspensions of diesel exhaust particles showed a time-dependent increase in the characteristic three peak EPR spectrum for a spin-adduct with the unpaired electron in the vicinity of a nitrogen atom (i.e., 4 oxo tempo, the oxidized form of Tempone-H) (Figure 3a). The signal increased at a constant rate over the 60 minute period. EPR measurements were approximately fivefold higher in suspensions that contained diesel filters (blank, 1,144 units; diesel, 5,864 units;  $t = 60$  min; Figure 3b). Superoxide dismutase (SOD) inhibited the EPR signal from diesel, causing a 30.2% reduction in signal. This concentration of SOD had a similar magnitude of effect on the diesel signal

TABLE 2. SYSTEMIC EFFECTS 24 HOURS AFTER EXPOSURE TO AIR AND DIESEL EXHAUST

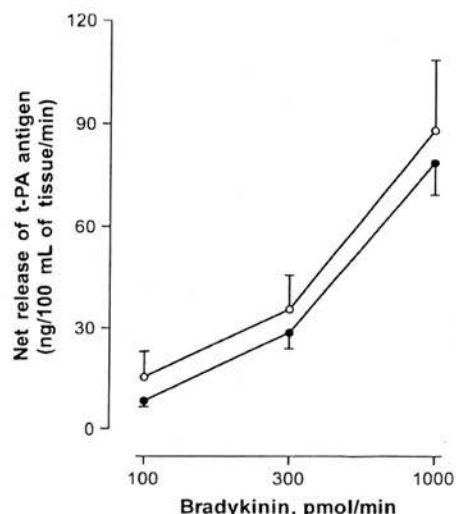
	Air	Diesel	Significance
Leukocytes, $\times 10^9$ cells/L	$5.2 \pm 0.4$	$5.2 \pm 0.3$	0.84
Neutrophils, $\times 10^9$ cells/L	$2.8 \pm 0.2$	$2.8 \pm 0.2$	0.57
IL-6, pg/ml	$1.5 \pm 0.2$	$2.2 \pm 0.2$	0.02
TNF- $\alpha$ , pg/ml	$0.88 \pm 0.07$	$0.99 \pm 0.07$	0.02
CRP, $\mu$ g/ml	$0.75 \pm 0.20$	$0.70 \pm 0.19$	0.97
sICAM-1, ng/ml	$171 \pm 6$	$181 \pm 8$	0.76
Nitrite, nM	$108.1 \pm 6.8$	$117.7 \pm 8.1$	0.45
TEAC, mM	$6.0 \pm 0.2$	$7.1 \pm 0.3$	0.003
sP-selectin, ng/ml	$33.7 \pm 1.8$	$36.5 \pm 1.4$	0.02
Platelets, $\times 10^9$ cells/L	$221 \pm 12$	$219 \pm 14$	0.57
t-PA antigen, ng/ml	$5.9 \pm 0.5$	$6.2 \pm 0.6$	0.63
PAI-1 antigen, ng/ml	$16.8 \pm 2.1$	$19.8 \pm 3.5$	0.45

Definition of abbreviations: CRP = C-reactive protein; IL-6 = interleukin-6; PAI-1 = plasminogen activator-1; sICAM-1 = soluble intercellular adhesion molecule 1; sP-selectin = soluble P-selectin; TEAC = Trolox equivalent antioxidant capacity; t-PA = tissue plasminogen activator; TNF- $\alpha$  = tumor necrosis factor- $\alpha$ . Values are reported as mean  $\pm$  SEM.

TABLE 3. FOREARM BLOOD FLOW AND PLASMA TISSUE PLASMINOGEN ACTIVATOR ANTIGEN CONCENTRATIONS

	Bradykinin (pmol/min)			
	0	100	300	1,000
Air				
FBF, ml/100 ml/min				
Noninfused arm	$2.2 \pm 0.2$	$2.3 \pm 0.2$	$2.2 \pm 0.1$	$2.6 \pm 0.3$
Infused arm	$4.8 \pm 1.0$	$12.3 \pm 1.4$	$14.8 \pm 1.5$	$20.2 \pm 2.0^*$
t-PA antigen, ng/ml				
Noninfused arm	$6.2 \pm 0.5$	$6.0 \pm 0.5$	$6.0 \pm 0.6$	$7.8 \pm 0.6$
Infused arm	$6.0 \pm 0.5$	$7.9 \pm 0.7$	$10.1 \pm 0.8$	$14.0 \pm 0.9^*$
Diesel				
FBF, ml/100 ml/min				
Noninfused arm	$2.6 \pm 0.3$	$2.6 \pm 0.2$	$2.5 \pm 0.2$	$2.5 \pm 0.3$
Infused arm	$4.1 \pm 0.7$	$10.7 \pm 1.0$	$13.8 \pm 1.1$	$18.8 \pm 1.2^*$
t-PA antigen, ng/ml				
Noninfused arm	$6.0 \pm 0.6$	$6.4 \pm 0.5$	$6.7 \pm 0.6$	$8.4 \pm 0.6$
Infused arm	$6.2 \pm 0.6$	$7.8 \pm 0.7$	$10.6 \pm 1.0$	$14.7 \pm 1.3^*$

Definition of abbreviations: FBF = forearm blood flow; t-PA = tissue plasminogen activator. Values are reported as mean  $\pm$  SEM. \*  $p < 0.001$ , analysis of variance (dose response).



**Figure 2.** Net release of tissue plasminogen activator (t-PA) antigen in subjects after diesel exposure (solid circles) and filtered air (open circles) during intrabrachial infusion of bradykinin.  $p < 0.001$  for both dose responses.  $p = 0.34$ , diesel exposure versus air.

as it did to that of the superoxide generator pyrogallol (34.7% reduction; Figure 3b).

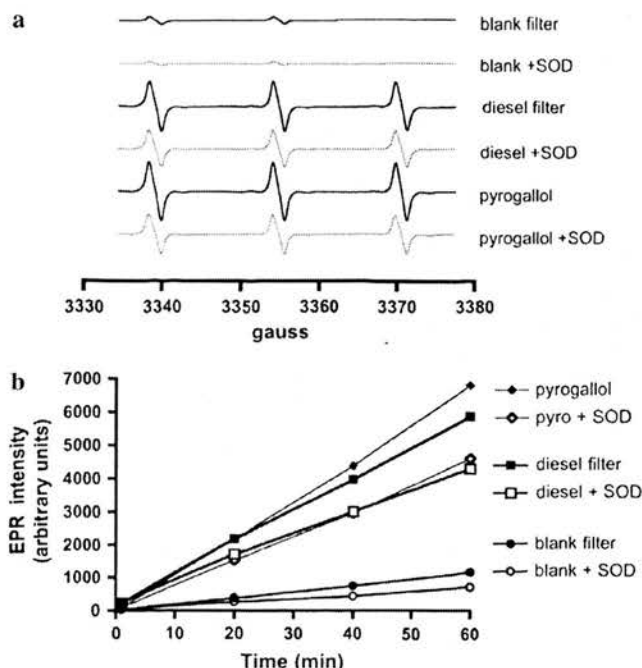
## DISCUSSION

Twenty-four hours after a transient 1-h exposure to diesel exhaust, there is a selective and persistent impairment of endothelium-dependent vasodilatation that occurs in the presence of mild systemic inflammation after a period of potential oxidative stress. These findings suggest that combustion-derived air pollution may have important adverse systemic and vascular effects for at least 24 hours after exposure. This may account for the epidemiologic observations of recent time-series studies suggesting both an acute (1–2 h) and a sustained peak in adverse cardiovascular outcomes after exposure to air pollution.

Exposure to air pollution causes airway inflammation and has an important negative effect on respiratory health. Diesel exhaust causes neutrophilic airway inflammation 6 to 24 hours after exposure (11, 12, 27), increases airway antioxidant defenses, and activates redox-sensitive transcription factors *in vivo*, consistent with oxidative stress-induced and cytokine-mediated inflammation (20, 28). It is increasingly recognized that these effects may induce important systemic effects (12, 29), including vascular inflammation (30).

We hypothesized that our initial observations of an immediate (within 2 h) impairment of vascular function were due to the oxidative effects of diesel exhaust (24). After exposure to diesel exhaust, endothelium-dependent and endothelium-independent nitric oxide donors caused reduced vasodilatation, whereas the endothelium- and nitric oxide-independent vasodilator verapamil caused normal vasodilatation. This pattern of vascular dysfunction suggested increased consumption of nitric oxide, whether it be endogenously derived from endothelial nitric oxide synthase or from an exogenous source, such as sodium nitroprusside.

In the current study, we demonstrate a persistent endothelium-dependent vascular dysfunction 24 hours after an hour-long exposure to diesel exhaust. Although vasodilatation to both endothelium-dependent agonists appeared to be impaired, this only



**Figure 3.** (a) Sample electron paramagnetic resonance (EPR) spectra generated from suspensions of blank and diesel particulate-coated filters, as well as the superoxide generator pyrogallol. EPR spectra were generated in the presence (grey lines) and absence (black lines) of superoxide dismutase (SOD). (b) Amplitude of EPR spectra (in arbitrary units) over a 60-minute incubation period in the presence (open symbols) and absence (closed symbols) of SOD. Diesel particulate (square symbols) caused a large increase in EPR intensity in comparison to suspension from the blank filter (circles). SOD caused an approximate 30% reduction in the EPR signal from diesel particulate and a similar reduction in signal to the superoxide generator pyrogallol (diamonds).

reached statistical significance for acetylcholine. Bradykinin causes vasodilatation through the release of various endothelium-derived factors, including nitric oxide, although it is believed that hyperpolarizing factor is the primary mediator of this response in humans (31). It is possible that differences in acetylcholine- and bradykinin-mediated vasodilatation may be explained by variation in the relative contribution of nitric oxide to the vasomotor response of these agonists. The mechanism of this selective impairment of endothelium-dependent vasodilatation has not been determined, but we suggest that this may be due to modification of endothelial homeostatic pathways after an initial oxidative burst.

The role of vascular oxidative stress in mediating endothelial dysfunction in this clinical model requires confirmation. However, the mechanism is supported by *in vitro* studies (32–34), as well as human exposure studies by our own group (25) and others (35).

The endothelium is a major target of oxidative stress and this interaction plays an important role in the pathophysiology of vascular disease (36). Incubation of aortic ring preparations with diesel exhaust particles inhibits acetylcholine-mediated relaxation, an effect that can be reversed by coincubation with the free radical scavenger SOD (32). Furthermore, diesel exhaust particles can induce oxidative modification of low-density lipoprotein, the major determinant of atheromatous vascular disease (37).



Diesel exhaust is a complex mixture of gases and particles, and from our findings we cannot exclude a role for nonparticulate or soluble components. The most abundant gaseous pollutants produced in the combustion of diesel fuel are oxides of nitrogen. In epidemiologic studies, ambient nitrogen dioxide (NO<sub>2</sub>) is considered a surrogate for traffic-derived pollution, with PM held responsible for the majority of the adverse health effects of air pollution (38). Although the direct effect of NO<sub>2</sub> on vascular function has not been studied to date, exposure to NO<sub>2</sub> alone at a higher concentration and for a longer duration than used here does not induce an inflammatory response in the airway mucosa (39). In contrast, exposure to dilute diesel exhaust for 1 hour at similar particulate concentrations to the present study resulted in pronounced airway mucosal inflammation with up-regulation of neutrophils, mast cells, and T lymphocytes (11). Although this suggests that the particulate phase of dilute diesel exhaust is responsible for the adverse inflammatory (11) and vascular effects of diesel exhaust (24), further proof is required with additional control exposures to nitrogen oxides and filtered diesel exhaust. Recent findings by Campen and colleagues, in which incubation with soluble components of diesel exhaust caused vascular dysfunction in isolated coronary artery rings from mice, highlight the need for further controlled exposure studies (40).

Whether diesel particulates or soluble components of the exposure, including organic hydrocarbons and transition metals, can directly affect the systemic vascular endothelium after inhalation also requires clarification. Although evidence that inhaled nanoparticles can translocate into the circulation in humans remains controversial (41, 42), it is not in doubt that diesel exhaust particulates are capable of inducing oxidative stress *in vitro*, with reactive oxidant species arising from the redox potential of the particles themselves and from the activation of inflammatory cells. Using EPR, we demonstrate that diesel exhaust particulate is capable of generating oxidative free radicals without prior interaction with pulmonary or vascular tissue. Furthermore, coincubation of diesel particles with SOD partially prevented this response, indicating a contribution of superoxide to this oxidative signal. However, measuring systemic oxidative stress *in vivo* is difficult, because the oxidative state is modulated by a range of antioxidant defenses (17). Interestingly, we demonstrate an increase in the antioxidant capacity of plasma 24 hours after exposure to diesel exhaust, perhaps suggesting up-regulation of antioxidant defense mechanisms after earlier systemic oxidative stress.

In contrast to our previous study, stimulated release of endothelial t-PA from the forearm circulation was not impaired at 24 hours (24). In health, the vascular endothelium delicately balances regulatory pathways controlling coagulation, fibrinolysis, and inflammation, as well as regulating vascular tone. It is perhaps not surprising that these complex dynamic functions are altered by exposure to diesel exhaust at different time points. Endogenous fibrinolytic function was impaired at 6 to 8 hours in our previous studies, but normalized at 24 hours, suggesting that this aspect of endothelial homeostasis recovers earlier than vasomotor function after exposure to air pollution.

We did not find evidence of a systemic cellular inflammatory response, but we did identify changes in proinflammatory cytokines IL-6 and TNF- $\alpha$ , raising the possibility that ongoing airway inflammation is contributing to the state of vascular dysfunction. Observational studies have strongly implicated systemic inflammation as a key pathological mechanism in the health effects of PM (7). In panel and population studies, increased PM exposure is associated with an acute-phase response with raised serum C-reactive protein concentrations (43), increased plasma viscosity (44), as well as altered hematologic indices (45) and plasma fibrinogen (46, 47). It is possible that, in a susceptible population,

in which inflammatory pathways may be up-regulated and anti-oxidant defenses may be depleted, an hour-long exposure to diesel exhaust would be sufficient to cause a greater systemic inflammatory response. Likewise, repeated exposure over a number of days or weeks may result in inflammation, with prolonged vascular dysfunction contributing to the pathogenesis of atherosclerosis. Indeed, in an apolipoprotein E-deficient (apoE<sup>-/-</sup>) mouse model, long term exposure to low concentrations of PM<sub>2.5</sub> altered vasomotor tone, induced vascular inflammation, and potentiated atherosclerosis (30).

Endothelial dysfunction, characterized as an impaired vasodilation to acetylcholine, predicts the likelihood of future cardiovascular events and death in patients with coronary artery disease (48) and in at risk individuals with normal coronary arteries (49). Although the mechanism of this association has not been precisely identified, this vascular dysfunction clearly has important clinical implications. Our findings of endothelial dysfunction 24 hours after diesel exhaust inhalation suggest that the adverse cardiovascular effects of combustion derived air pollution are mediated through persistent detrimental vascular effects.

## Conclusions

In healthy volunteers, inhalation of diesel exhaust for 1 hour, at particle concentrations encountered in an urban setting, causes mild systemic inflammation and an impairment of vascular endothelial function that persisted for up to 24 hours after the exposure. This occurred in the absence of alterations in endogenous fibrinolytic capacity. These findings provide a plausible explanation for the observed increase in acute cardiovascular events 24 hours after a peak in traffic-related PM air pollution.

**Conflict of Interest Statement:** None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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## **SECTION 3**

### **ISCHAEMIC HEART DISEASE (Publications 17-25)**

**PUBLICATION 17**

# Impaired Coronary Tissue Plasminogen Activator Release Is Associated With Coronary Atherosclerosis and Cigarette Smoking

## Direct Link Between Endothelial Dysfunction and Atherothrombosis

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**Background**—The aim of the study was to establish the influence of proximal coronary artery atheroma and smoking habit on the stimulated release of tissue plasminogen activator (tPA) from the heart.

**Methods and Results**—After diagnostic coronary angiography in 25 patients, the left anterior descending coronary artery (LAD) was instrumented, and the proximal LAD plaque volume was determined by use of intravascular ultrasound (IVUS). Blood flow and fibrinolytic responses to selective LAD infusion of saline, substance P (10 to 40 pmol/min; endothelium-dependent), and sodium nitroprusside (5 to 20  $\mu$ g/min; endothelium-independent) were measured by intracoronary IVUS and Doppler, combined with arterial and coronary sinus blood sampling. Mean plaque burden was  $5.5 \pm 0.8$  mm<sup>3</sup>/mm vessel (range 0.6 to 13.7 mm<sup>3</sup>/mm vessel). LAD blood flow increased with both substance P and sodium nitroprusside ( $P < 0.001$ ), although coronary sinus plasma tPA antigen and activity concentrations increased only during substance P infusion ( $P < 0.006$  for both). There was a strong inverse correlation between the LAD plaque burden and release of active tPA ( $r = -0.61$ ,  $P = 0.003$ ). Cigarette smoking was associated with impaired coronary release of active tPA (current smokers,  $31 \pm 23$  IU/min; ex-smokers,  $50 \pm 33$  IU/min; nonsmokers  $202 \pm 73$  IU/min;  $P < 0.05$ ).

**Conclusions**—We found that both the coronary atheromatous plaque burden and smoking habit are associated with a reduced acute local fibrinolytic capacity of the heart. These important findings provide evidence of a direct link between endogenous fibrinolysis, endothelial dysfunction, and atherothrombosis in the coronary circulation and may explain the greater efficacy of thrombolytic therapy for myocardial infarction in cigarette smokers. (*Circulation*. 2001;103:1936-1941.)

**Key Words:** thrombolysis ■ endothelium ■ coronary disease ■ ultrasonics

The fibrinolytic factor tissue plasminogen activator (tPA) is a serine protease that regulates the degradation of intravascular fibrin and is released from the endothelium through the translocation of a dynamic intracellular storage pool. If endogenous fibrinolysis is to be effective, then the rapid mobilization of tPA from the endothelium is essential, because thrombus dissolution is much more effective if tPA is incorporated during, rather than after, thrombus formation.<sup>1</sup> The efficacy of plasminogen activation and fibrin degradation is further determined by the relative balance between the acute local release of tPA and its subsequent inhibition through formation of complexes with the serpin, plasminogen activator inhibitor type 1 (PAI-1). This dynamic aspect of endothelial function and fibrinolytic balance may be di-

rectly relevant to the pathogenesis of atherothrombosis, but only recently have robust methods to determine acute tPA release been developed.<sup>2</sup>

Small areas of denudation and thrombus deposition are a common finding on the surface of atheromatous plaques and are usually subclinical.<sup>3</sup> In the presence of an imbalance in the fibrinolytic system, however, such microthrombi may propagate, ultimately leading to arterial occlusion.<sup>4</sup> Indeed, in genetic murine models, tPA deficiency is associated with myocardial necrosis and the development of regional wall motion abnormalities.<sup>5</sup> Recently, Rosenberg and Aird<sup>4</sup> postulated that vascular bed-specific defects in hemostasis exist and that coronary thrombosis critically depends on the local fibrinolytic balance. To date, however, no clinical studies have directly assessed the acute local fibrinolytic capacity of

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the coronary vascular bed in patients with coronary artery disease.

Using forearm venous occlusion plethysmography and the endothelium-dependent agonist substance P, we recently characterized a new model of assessing the acute release of endogenous tPA *in vivo* in humans.<sup>2</sup> This has allowed us to show that cigarette smoking is associated with an impairment of acute tPA release in the forearm circulation.<sup>6</sup> We hypothesized that the acute local coronary release of tPA would be influenced by both the extent of coronary atheroma and smoking habit. Therefore, the aims of the present study were first, to apply this approach to the coronary circulation and thereby establish a method of assessing acute coronary tPA release; second, to determine the relationship between the extent of coronary artery atheroma, quantified by intravascular ultrasound (IVUS), and the acute fibrinolytic capacity of the coronary vascular bed; and third, to show whether cigarette smoking impairs coronary, as well as forearm, tPA release.

## Methods

### Patient Selection

Patients were excluded if they had significant left main stem disease or a minimal luminal diameter of <2 mm in the proximal left anterior descending coronary artery (LAD). Coronary risk factors were determined in all patients by standard clinical criteria. The study was undertaken with the approval of the local research ethics committee, in accordance with the Declaration of Helsinki, and with the written informed consent of each subject.

### Study Protocol

All patients discontinued their medication on the study day, attended in the fasting state, and underwent diagnostic coronary angiography at 8 AM. The coronary sinus was cannulated from the femoral vein with a preformed specific 6F catheter (Torcon NB catheter, Cook)<sup>7</sup> that was advanced beyond the posterior interventricular vein. Stable and selective cannulation of the coronary sinus was achieved in all but 3 subjects. Arterial samples were obtained through an 8F hemostatic sheath placed in the right femoral artery.

The left coronary artery was cannulated with a 7F guiding catheter, and a 0.014-in 12.5-MHz Doppler wire (FloWire, Cardiometrics, Endosonics) was passed into the LAD. A 3.2F Ultracross 20-MHz IVUS imaging catheter (Scimed, Boston Scientific Corp) was advanced into the LAD over the Doppler wire. The IVUS examination of the proximal artery was performed at 0.5 mm/s with a motorized pullback device (Boston Scientific Corp). After the pullback examination, the IVUS imaging catheter was repositioned just distal to the ostium of the LAD. The Doppler guidewire was retracted to the tip of the imaging catheter and maintained in a stable position by the short monorail segment of the IVUS catheter.<sup>8</sup>

### Drug Administration

Pharmaceutical-grade substance P (Clinalfa AG), an endothelium-dependent vasodilator, and sodium nitroprusside (David Bull Laboratories), an endothelium-independent vasodilator, were administered after dissolution in saline. Five-minute infusions were administered at 1 mL/min via the IVUS catheter flush port.<sup>8</sup> The agents were given in the following order: saline, substance P 10 pmol/mL, substance P 20 pmol/mL, substance P 40 pmol/mL, sodium nitroprusside 5 µg/mL, and sodium nitroprusside 20 µg/mL.

### Measurement of Plaque Volume and Coronary Blood Flow

Computerized 3D reconstructions of the proximal LAD were performed offline by a single blinded operator using the TomTec

computer system (Echoscan, TomTec Imaging Systems). The proximal atheromatous plaque volume was calculated with a well-validated edge-detection algorithm as previously described.<sup>9,10</sup>

The LAD cross-sectional area was measured by computerized planimetry (Clearview, Boston Scientific Inc) of the vessel lumen at the onset of the QRS complex. Blood flow velocity was determined by use of average peak velocity of the Doppler signal (FloMap, Cardiometrics). Blood flow in the LAD was previously defined as half the product of the average peak velocity and the cross-sectional area<sup>11</sup> and was determined from the mean of 5 measurements made in the final minute of each infusion period.

### Blood Sampling and Plasma Assays

Ten milliliters of arterial and 10 mL of coronary sinus blood were obtained simultaneously at the end of each infusion period and collected into acidified buffered citrate (Biopool Stabilyte) and citrate (Monovette, Sarstedt) tubes, and platelet-free plasma was decanted and stored at -80°C before assay. Coronary sinus oxygen saturations were determined at the end of each infusion period with an automated oximeter (Oximax 300, Watco Services). Plasma tPA

TABLE 1. Patient Characteristics

No.	25
Sex, male	17
Age, y	56±2
Body mass index, kg/m <sup>2</sup>	28±1
Risk factors, n	
Current/ex-smoker	6/9
Hypertension	6
Diabetes mellitus	3
Hyperlipidemia	19
Family history	13
Serum lipid profile, mg/dL	
Total cholesterol	221±10
LDL cholesterol	121±7
HDL cholesterol	51±3
Total/HDL cholesterol ratio	4.9±0.5
Triglycerides	204±8
Medical therapy, n	
Aspirin	22
β-Adrenergic blockade	18
Lipid-lowering therapy	16
Calcium antagonism	12
Long-acting nitrate	8
ACE inhibition	3
Diuretics	3
Previous myocardial infarction, n	6
Noninvasive testing, n	
Low risk	9
High risk	12
Not performed	4
Angiographic data, n	
Good left ventricular function	24
Normal/mild disease	8
Single-vessel disease	9
2-Vessel disease	6
3-Vessel disease	2

and PAI-1 antigen and activity concentrations were determined with ELISAs and a photometric method as previously described.<sup>2,6</sup>

### Data Analysis and Statistics

Coronary tPA release was defined as the product of the LAD plasma flow and the plasma arterial and coronary sinus concentration differences. To compare vasomotor and fibrinolytic responses with proximal atheromatous plaque volume, the area under the curve (AUC) was calculated for each response: coronary blood flow, plasma arterial and coronary sinus tPA concentration differences, and estimated net tPA release.

Data were examined by ANOVA with repeated measures, Student's *t* test, and univariate and multivariate regression analysis with StatView v5.0.1 (SAS Institute Inc). Where ANOVA demonstrated significant differences in responses, post hoc comparisons were made by use of the Fisher protected least significant difference test (StatView v5.0.1). Multivariate regression analysis was performed only on those factors that were shown to have a significant association by univariate analysis. All results are expressed as mean  $\pm$  SEM. Statistical significance was taken at the 5% level.

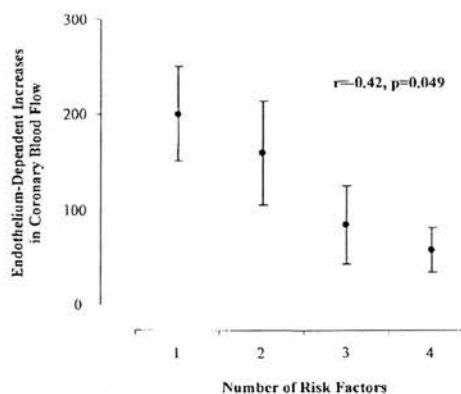
### Results

Baseline patient characteristics are shown in Table 1. In keeping with the anticipated profile of patients undergoing coronary angiography, the study population was predominantly male and middle-aged and had a combination of risk factors. Throughout the study, there were no significant changes in heart rate, mean arterial pressure, or hematocrit ( $0.40 \pm 0.01$ ).

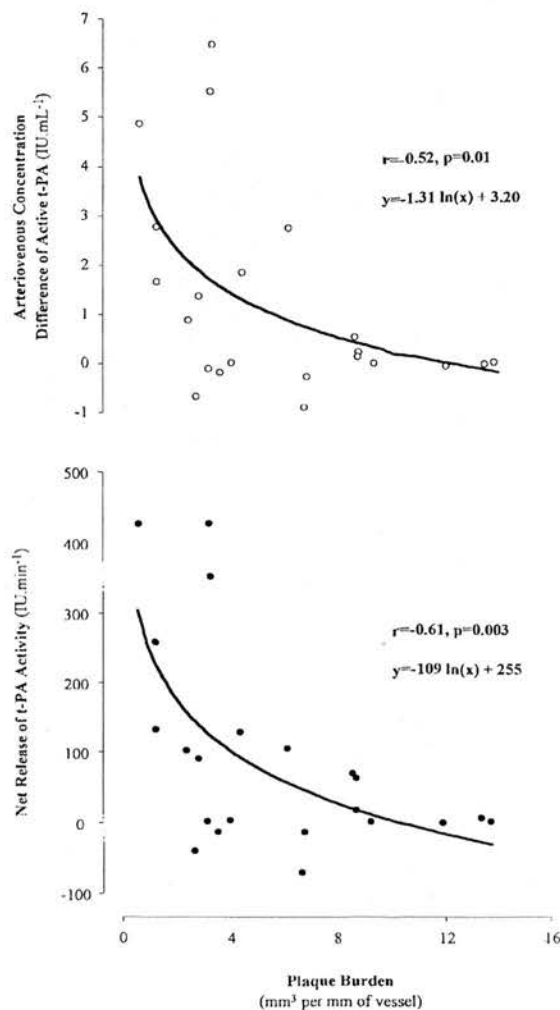
#### Plaque Volume and Blood Flow Responses

The proximal  $29 \pm 1$  mm of the LAD was reconstructed and found to contain  $160 \pm 24$  mm<sup>3</sup> of atheromatous plaque: a plaque burden of  $5.5 \pm 0.8$  mm<sup>3</sup>/mm vessel (range, 0.6 to 13.7 mm<sup>3</sup>/mm vessel). There was a significant linear correlation between the plaque burden and the serum total cholesterol:HDL cholesterol ratio ( $r=0.55$ ,  $P=0.004$ ).

LAD blood flow increased with both substance P and sodium nitroprusside infusion ( $P<0.001$ , ANOVA; see Table 2). There was a significant linear correlation between the percentage increase in coronary sinus oxygen saturations and LAD flow ( $r=0.46$ ,  $P<0.001$ ). There was no correlation, however, between the plaque burden and the AUC for the coronary blood flow responses to substance P or sodium nitroprusside infusion. In contrast, there was an association



**Figure 1.** Prevalence of risk factors and influence on coronary blood flow responses to substance P infusion.



**Figure 2.** Correlation of plaque burden of LAD with AUC for arteriovenous concentration differences (top) and estimated net release (bottom) of tPA activity.

between the number of risk factors for atherosclerosis and the coronary blood flow responses to substance P (Figure 1:  $r=-0.42$ ,  $P<0.05$ ).

#### Plasma Fibrinolytic Parameters

There was a significant increase in plasma tPA antigen and activity concentrations from the coronary sinus during substance P infusion (Table 2: ANOVA,  $P<0.001$  and  $P<0.006$ , respectively) but not during sodium nitroprusside infusion. There was a significant inverse correlation between the plaque burden and the AUC for active tPA release (Figure 2:  $r=-0.61$ ,  $P=0.003$ ) and a trend for the AUC for tPA antigen release ( $r=-0.34$ ,  $P=0.15$ ). There was also an inverse linear correlation between the basal coronary sinus plasma tPA antigen concentration and the AUC for active tPA release ( $r=-0.58$ ,  $P<0.005$ ).

Current smokers had a higher basal plasma tPA antigen concentration despite similar plasma PAI-1 concentrations



**TABLE 2. Hemodynamics, Coronary Blood Flow, and tPA and PAI-1 Concentrations During Substance P and Sodium Nitroprusside Infusion**

	Substance P, pmol/min				Sodium Nitroprusside, $\mu$ g/min		
	Saline	10	20	40	Saline	5	20
Heart rate, bpm	65 $\pm$ 2	64 $\pm$ 3	64 $\pm$ 3	64 $\pm$ 3	69 $\pm$ 3	69 $\pm$ 3	68 $\pm$ 4
Mean arterial pressure, mm Hg	98 $\pm$ 3	99 $\pm$ 3	98 $\pm$ 3	98 $\pm$ 3	94 $\pm$ 3	96 $\pm$ 5	90 $\pm$ 4
Coronary sinus oxygen saturation, %	44 $\pm$ 2	48 $\pm$ 2	48 $\pm$ 3	46 $\pm$ 2	44 $\pm$ 3	...	54 $\pm$ 4
IVUS and Doppler							
Luminal cross-sectional area, mm <sup>2</sup>	15.7 $\pm$ 1.0	16.4 $\pm$ 1.0†	16.5 $\pm$ 1.0†	16.9 $\pm$ 1.0† §	14.9 $\pm$ 1.1	15.4 $\pm$ 1.1*	16.1 $\pm$ 1.3†
Average peak velocity, cm/s	21.4 $\pm$ 1.6	27.3 $\pm$ 2.2†	27.5 $\pm$ 2.4†	28.0 $\pm$ 2.3† §	22.7 $\pm$ 2.2	27.0 $\pm$ 2.2†	47.1 $\pm$ 4.6† §
Absolute coronary blood flow, mL/min	103 $\pm$ 12	138 $\pm$ 18†	140 $\pm$ 19†	143 $\pm$ 18† §	109 $\pm$ 18	131 $\pm$ 20†	242 $\pm$ 42† §
Change in coronary blood flow, %	0	36 $\pm$ 6†	37 $\pm$ 7†	46 $\pm$ 10† §	0	29 $\pm$ 9†	140 $\pm$ 24† §
Plasma tPA antigen, ng/mL							
Coronary sinus concentration	7.9 $\pm$ 0.5	8.8 $\pm$ 0.7†	8.8 $\pm$ 0.6†	9.1 $\pm$ 0.7† §	8.1 $\pm$ 1.0	...	8.7 $\pm$ 1.0
Arterial concentration	7.7 $\pm$ 0.5	8.0 $\pm$ 0.5	8.1 $\pm$ 0.6	8.1 $\pm$ 0.6	8.1 $\pm$ 1.0	...	8.9 $\pm$ 1.3
Arteriovenous concentration difference	0.3 $\pm$ 0.2	0.8 $\pm$ 0.3	0.7 $\pm$ 0.3	1.0 $\pm$ 0.3*	0.0 $\pm$ 0.2	...	-0.1 $\pm$ 0.4
Plasma tPA activity, IU/mL							
Coronary sinus concentration	0.5 $\pm$ 0.1	1.1 $\pm$ 0.3†	0.8 $\pm$ 0.2	1.0 $\pm$ 0.2† §	0.7 $\pm$ 0.2	...	0.7 $\pm$ 0.2
Arterial concentration	0.4 $\pm$ 0.1	0.5 $\pm$ 0.1	0.6 $\pm$ 0.2	0.7 $\pm$ 0.2† §	0.5 $\pm$ 0.1	...	0.8 $\pm$ 0.3
Arteriovenous concentration difference	0.1 $\pm$ 0.1	0.6 $\pm$ 0.2†	0.2 $\pm$ 0.1	0.3 $\pm$ 0.2	0.2 $\pm$ 0.1	...	-0.0 $\pm$ 0.0
Plasma PAI-1 antigen, ng/mL							
Coronary sinus concentration	70 $\pm$ 7	70 $\pm$ 7	68 $\pm$ 7	68 $\pm$ 7	68 $\pm$ 11	...	67 $\pm$ 12
Arterial concentration	71 $\pm$ 8	70 $\pm$ 8	67 $\pm$ 7	67 $\pm$ 7	70 $\pm$ 12	...	67 $\pm$ 12
Arteriovenous concentration difference	-0 $\pm$ 2	-1 $\pm$ 1	1 $\pm$ 1	0 $\pm$ 1	-1 $\pm$ 1	...	0 $\pm$ 1
Plasma PAI-1 activity, AU/mL							
Coronary sinus concentration	21 $\pm$ 4	21 $\pm$ 3	19 $\pm$ 4	17 $\pm$ 3	24 $\pm$ 8	...	23 $\pm$ 9
Arterial concentration	20 $\pm$ 3	19 $\pm$ 3	20 $\pm$ 3	19 $\pm$ 3	26 $\pm$ 1	...	31 $\pm$ 6
Arteriovenous concentration difference	1 $\pm$ 1	1 $\pm$ 1	-0 $\pm$ 0	-2 $\pm$ 1	1 $\pm$ 1	...	0 $\pm$ 0

AU indicates arbitrary units. n=14 for sodium nitroprusside responses; n=22 for fibrinolytic parameters (except PAI-1 activity; n=13).

\* $P\leq 0.05$ ; † $P\leq 0.01$ ; ‡ $P\leq 0.001$ , Fisher's protected least significant difference test (vs baseline).

§ $P\leq 0.007$ ; || $P\leq 0.05$ , ANOVA with repeated measures.

and coronary arterial plaque burden (Table 3). Current and ex-smokers released significantly less active tPA than non-smokers (Figure 3; ANOVA,  $P<0.05$ ). Hypercholesterolemia, hypertension, diabetes mellitus, and a family history of premature coronary artery disease did not appear to influence active tPA release, although some of the subgroup sample sizes were small.

There were no significant changes in plasma PAI-1 antigen and activity concentrations throughout the study (Table 2). Basal coronary sinus plasma PAI-1 antigen concentrations correlated positively with plaque burden ( $r=0.47$ ,  $P<0.03$ ) and negatively with release of active tPA ( $r=-0.44$ ,  $P=0.04$ ).

Multivariate regression analysis identified plaque burden and basal coronary sinus tPA antigen concentrations as the independent variables that were significantly associated with release of active tPA ( $P\leq 0.02$  for both).

### Discussion

For the first time, we have shown a direct relationship between both the coronary atheromatous plaque burden and smoking habit and the acute stimulated fibrinolytic capacity of the heart. These important findings suggest that both

atherosclerosis and smoking habit adversely influence the local fibrinolytic balance in the coronary circulation and provide a direct link between endothelial dysfunction, atherothrombosis, and myocardial infarction.

This is the first clinical study to attempt to directly assess the acute release of tPA in the coronary circulation and to have found it to be sensitive to the presence of atheroma: a rapid decline in release of active tPA associated with an increasing plaque burden. The reduction in acute fibrinolytic capacity appears to reflect both an impairment of acute tPA release and an elevation of plasma PAI-1 concentrations. The mechanisms underlying this relationship remain to be established but are likely to involve chronic endothelial cell injury and possibly an impairment of the L-arginine-nitric oxide pathway.<sup>12</sup> In addition, this association may reflect a chronic stimulation and upregulation of basal tPA release caused by arterial denudation and atheroma.<sup>13</sup> The subsequent depletion of endothelial cell tPA stores, the associated increases in PAI-1 concentrations, and the overall reduction of the acute dynamic fibrinolytic response would potentially limit the capacity of the vasculature to lyse intraluminal thrombus. This is consistent with the epidemiological observations of a positive correlation between plasma tPA and PAI-1 antigen

concentrations and future coronary events,<sup>7,14,15</sup> as well as our findings of an inverse correlation of active tPA release with basal coronary sinus tPA and PAI-1 antigen concentrations.

Questions of cause and effect cannot be resolved by the present study. Indeed, our observations are consistent with a reduced fibrinolytic activity causing enhanced atherogenesis. Detailed postmortem studies have shown that plaque growth is induced by episodic subclinical plaque disruption and thrombus formation.<sup>16</sup> The prolonged presence of residual thrombus over a disrupted or eroded plaque will provoke smooth muscle migration and the production of new connective tissue, leading to plaque expansion.<sup>3,17</sup> This is consistent with the enhanced macrovascular fibrin deposition and atherogenesis seen in genetic murine models of tPA and plasminogen deficiency.<sup>5,18</sup> It is likely, however, that both processes, impaired fibrinolysis and atherogenesis, cooperate and interact to damage vascular function and structure.

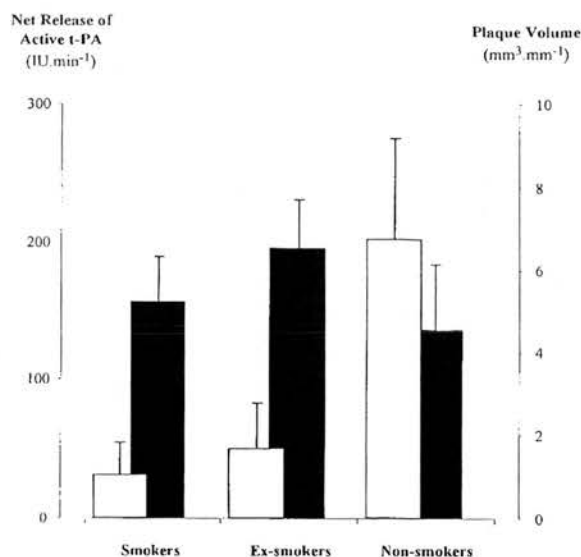
Consistent with our previous work in the peripheral circulation,<sup>6</sup> we have observed an elevated basal plasma tPA antigen concentration and an impaired coronary release of active tPA in cigarette smokers. These observations suggest that impaired endogenous fibrinolysis may contribute to the increased risk of coronary thrombosis seen in smokers through propagation of thrombus that would otherwise undergo lysis and remain subclinical. Although cigarette smokers have a higher overall mortality from myocardial infarction than nonsmokers, the in-hospital mortality has consistently been shown to be lower. This so-called "smokers' paradox" can be explained by the observation that the infarct-related artery is more than twice as likely to become patent in current smokers than in nonsmokers after thrombolytic therapy for acute myocardial infarction.<sup>19</sup> Indeed, it has been provocatively suggested that thrombolytic therapy should be given only to smokers and that such alternative strategies as

primary angioplasty be used in nonsmokers.<sup>20</sup> Our findings may account for these observations, because it might be anticipated that patients with impaired coronary endothelial cell tPA release would benefit most from thrombolytic therapy.

Quantitative coronary angiography<sup>21</sup> has suggested that there is a direct association between coronary atherosclerosis and endothelium-dependent vasodilation. Quantitative coronary angiography, however, has several inherent limitations and inaccuracies<sup>22</sup> that occur because it can assess only the arterial lumen and is unable to take account of "Glagovian" arterial remodeling.<sup>23</sup> In contrast, IVUS provides a more accurate assessment of intracoronary plaque volume that has been extensively validated.<sup>9,10</sup> Using this methodology, we did not find an association between the atherosclerotic plaque burden and the magnitude of the substance P-induced vasodilation. This is, in part, likely to reflect the independent influence of atherosclerotic risk factors on endothelium-dependent vasomotion and is borne out by the correlation of the vasodilation to substance P with the prevalence of these risk factors.

### Study Limitations

This study was conducted in the necessary clinical setting of patients with a combination of risk factors and concomitant therapies undergoing diagnostic coronary angiography. The modest sample size means that this study lacks sufficient power to address the influence of all the individual variables associated with coronary artery disease. In particular, because the number of patients with diabetes mellitus or normocholesterolemia was small, our study may have failed to detect potential associations between these factors. Moreover, it is difficult to assess the effect of hypercholesterolemia and hypertension given the high incidence of treatment with



**Figure 3.** Influence of smoking status on AUC for net release of active tPA (open bars) and plaque volume (solid bars).  $P < 0.05$  ANOVA; \* $P = 0.03$  Fisher's protected least significant difference test, current or ex-smokers vs nonsmokers.

**TABLE 3.** Influence of Smoking Status on Plaque Burden and Basal Plasma tPA and PAI-1 Concentrations

	Current Smokers (n=6)	Ex-Smokers (n=9)	Nonsmokers (n=7)
Plaque burden, mm <sup>3</sup> /mm	5.2±1.1	6.5±1.2	4.5±1.6
Plasma tPA antigen, ng/mL			
Coronary sinus concentration	9.4±1.3†	8.2±0.4	6.3±0.7*
Arterial concentration	9.0±1.4†	7.8±0.3	6.3±0.8*
Plasma tPA activity, IU/mL			
Coronary sinus concentration	0.3±0.1	0.6±0.2	0.6±0.2‡
Arterial concentration	0.3±0.0	0.5±0.1	0.5±0.2‡
Plasma PAI-1 antigen, ng/mL			
Coronary sinus concentration	88±15	67±9	59±14
Arterial concentration	84±15	70±11	60±15
Plasma PAI-1 activity, AU/mL			
Coronary sinus concentration	14±6	25±5	24±8
Arterial concentration	12±5	26±4	23±8

AU indicates arbitrary units.

\* $P < 0.04$ ; † $P = 0.06$ , ANOVA with repeated measures.

‡ $P = 0.01$ , Fisher's protected least significant difference test (nonsmokers vs current smokers).

lipid-lowering and antihypertensive therapy. Given the concordance between our previous findings in the forearm circulation of smokers,<sup>6</sup> however, the influence of such risk factors as diabetes mellitus and hypercholesterolemia may be more readily assessed in the peripheral circulation.

In conclusion, we have demonstrated, for the first time, a direct association between both the coronary atheromatous plaque burden and smoking habit with the acute local fibrinolytic capacity of the coronary circulation. These important findings may provide the main link between endothelial dysfunction and atherothrombosis, as well as an explanation for the smokers' paradox. Interventions aimed at the enhancement of the local coronary fibrinolytic capacity could potentially be of major clinical importance.

### Acknowledgments

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**PUBLICATION 18**



## Regular Article

## Preserved endothelial vasomotion and fibrinolytic function in patients with acute stent thrombosis or in-stent restenosis

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## Abstract

**Introduction:** Acute stent thrombosis and in-stent restenosis are serious complications of percutaneous coronary intervention (PCI) and may be associated with vascular or platelet abnormalities. We aimed to assess endothelium-dependent vasomotion, endogenous fibrinolysis and platelet function in patients with acute stent thrombosis or in-stent restenosis. **Materials and methods:** Thirty-six subjects were enrolled into four groups: acute stent thrombosis, in-stent restenosis, uncomplicated PCI with stent implantation and healthy matched controls. Forearm blood flow was measured using bilateral venous occlusion plethysmography during intra-brachial acetylcholine, substance P and sodium nitroprusside infusion. Venous blood samples were withdrawn for estimation of plasma fibrinolytic variables and platelet aggregometry. **Results:** Acetylcholine, substance P and sodium nitroprusside caused dose-dependent increases in blood flow ( $P < 0.001$ ) and substance P caused a dose-dependent increase in tissue-type plasminogen activator (t-PA) release ( $P < 0.001$ ) in all groups. Thrombin, collagen, adenosine diphosphate (ADP) and the thromboxane A2 analogue, U46619, caused dose-dependent platelet aggregation ( $P < 0.001$ ) in all groups. There were no significant between group differences in these responses except that, in keeping with aspirin therapy, collagen-induced platelet aggregation was impaired in patient groups compared with healthy controls ( $P < 0.01$ ). Post-hoc analysis demonstrated a significant impairment of acute t-PA release in current smokers compared to non-smokers ( $P < 0.05$ ). **Conclusions:** Despite previous reports suggesting impaired vascular function, endothelium-dependent vasomotion, endogenous fibrinolysis and platelet aggregation do not appear to play a major role in the pathogenesis of acute stent thrombosis or in-stent restenosis.

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**Keywords:** Endothelial function; Platelets; In-stent restenosis; Stent thrombosis; Fibrinolysis

Coronary artery stent implantation is a valuable adjunct to percutaneous transluminal coronary angioplasty. It reduces the absolute incidence of restenosis compared with balloon angioplasty by 10–15% and improves 6-month event free survival by 10–20% [1,2]. However, there is a small but significant risk of acute coronary stent thrombosis [3] and in-stent restenosis that can have devastating consequences

including myocardial infarction and death [4]. Whilst procedure-related complications such as persistent dissection, longer stent length and final minimal lumen diameter may be implicated in some cases of stent thrombosis or restenosis [3], no underlying precipitant can be identified in many patients.

Stent thrombus formation is principally initiated by platelet aggregation, which, in the absence of effective endothelium-derived vasoregulation and fibrinolysis, is then stabilised by the deposition of a fibrin mesh. The chronology of in-stent restenosis has been described as early thrombosis, followed by thrombus endothelialization and infiltration by lymphocytes and monocytes, and finally smooth muscle cell migration and proliferation within the resolving thrombus [5–7]. The initiation, propagation and

**Abbreviations:** ADP, adenosine diphosphate; ANOVA, analysis of variance; AUC, area under the curve; PAI-1, plasminogen activator inhibitor type 1; PCI, percutaneous coronary intervention; t-PA, tissue-type plasminogen activator.

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stabilization of acute stent thrombosis and in-stent restenosis are therefore dependent on several components: platelet aggregation, endothelial function, coagulation and fibrinolysis.

The purpose of the present study was to identify potential factors that may be implicated in the predisposition to acute stent thrombosis or in-stent restenosis using a case-control methodology. We assessed endothelium-dependent vasomotion, platelet aggregation and the acute endogenous fibrinolytic capacity in patients who have developed thrombotic complications following percutaneous coronary intervention (PCI) and stent implantation.

## 1. Materials and methods

### 1.1. Subjects

Twenty-six patients who had undergone PCI with stent implantation at least 6 months previously and 10 age- and sex-matched healthy control subjects were recruited into the study. In the patient group, 16 had developed complications of acute stent thrombosis within 48 h of stent implantation ( $n=6$ ) or in-stent restenosis within 6 months of intervention ( $n=10$ ), and 10 had no clinical evidence of stent thrombosis or restenosis at least 1 year following the procedure. Patients with procedural complications or suboptimal stent insertion were excluded. All patients received thienopyridine therapy for 4 weeks after undergoing PCI and none received a glycoprotein IIb/IIIa receptor antagonist during the procedure.

All subjects abstained from alcohol for 24 h, and from food and caffeine-containing drinks for at least 4 h before the study, and medications were withheld on the day of the study. All studies were performed in a quiet, temperature-controlled room maintained at 22–25 °C. The investigation was performed with the approval of the local ethics committee and conformed with the principles outlined in the Declaration of Helsinki, and with the written informed consent of each subject.

### 1.2. Drugs administration

Pharmaceutical-grade substance P (Clinalfa, Läufelfingen, Switzerland), acetylcholine (Cibavision Ophthalmics, Southampton, UK) and sodium nitroprusside (David Bull Laboratories, Faulding, UK) were administered following dissolution in saline (0.9%: Baxter Healthcare). All solutions were freshly prepared on the day of study.

### 1.3. Hemodynamic measurements

Blood flow was measured in both forearms by venous occlusion plethysmography using mercury-in-silastic strain gauges as previously described [8,9]. Blood pres-

sure was monitored in the non-infused arm at intervals throughout each study with a semiautomated noninvasive oscillometric sphygmomanometer (Takeda UA 751, Takeda Medical).

### 1.4. Fibrinolytic assays

Venous blood (10 ml) was withdrawn simultaneously from each arm and collected into acidified buffered citrate (Biopool® Stabilyte™, Umeå, Sweden; for t-PA assays) and trisodium citrate (Monovette®, Sarstedt, Nümbrecht, Germany; for PAI-1 assays) tubes and centrifuged at  $2000 \times g$  for 30 min at 4 °C. Platelet-free plasma was decanted and stored at –80 °C before assay. Plasma tissue-type plasminogen activator (t-PA) and plasminogen activator inhibitor type 1 (PAI-1) antigens were determined as previously described [8] using enzyme-linked immunosorbent assays (Coaliza t-PA and Coaliza PAI-1, Chromogenix, Mölndal, Sweden) and plasma t-PA activity using a photometric method (Coatest t-PA, Chromogenix).

### 1.5. Platelet aggregometry and thrombophilia screen

Fasting venous blood (30 ml) was collected into trisodium citrate tubes (Monovette®) and immediately centrifuged at  $120 \times g$  for 10 min to obtain platelet-rich plasma, which was aspirated, adjusted to give a platelet count of  $250 \times 10^9/l$  and pre-warmed to 37 °C. Aggregation studies were performed

Table 1  
Baseline characteristics

	Acute stent thrombosis ( $n=6$ )	In-stent restenosis ( $n=10$ )	Control patients ( $n=10$ )	Healthy subjects ( $n=10$ )
Sex (male/female)	2:4	9:1	8:2	9:1
Age	$65 \pm 3$	$62 \pm 2$	$63 \pm 2$	$58 \pm 3$
Current smoker, % ( $n$ )	17 (1)	20 (2)	20 (2)	20 (2)
Diabetes mellitus, % ( $n$ )	17 (1)	30 (3)	20 (2)	0 (0)
Hypertension, % ( $n$ )	50 (3)	60 (6)	30 (3)	0 (0)
Three-vessel disease, % ( $n$ )	33 (2)	30 (3)	20 (2)	0 (0)
Normal or mild left ventricular impairment, % ( $n$ )	84 (5)	90 (9)	80 (8)	100 (10)
Average stent diameter, mm	$3.3 \pm 0.2$	$3.0 \pm 0.1$	$3.2 \pm 0.2$	
Average stent length, mm	$16 \pm 2$	$18 \pm 2$	$20 \pm 2$	
<i>Medical therapy</i>				
Aspirin, % ( $n$ )	100 (6)	100 (10)	100 (10)	0 (0)
$\beta$ -adrenoceptor antagonists, % ( $n$ )	50 (3)	50 (5)	60 (6)	0 (0)
Angiotensin-converting enzyme inhibitors, % ( $n$ )	33 (2)	60 (6)	40 (4)	0 (0)
Statins, % ( $n$ )	100 (6)	80 (8)	80 (8)	0 (0)
Calcium channel blockers, % ( $n$ )	50 (3)	60 (6)	10 (1)	0 (0)
Oral nitrates, % ( $n$ )	50 (3)	30 (3)	20 (2)	0 (0)
Mean $\pm$ S.E.M.				

Table 2  
Infused forearm blood flow during substance P, acetylcholine and sodium nitroprusside infusion

Drug infusion	Infused forearm blood flow (ml/100 ml/min)			
	Acute stent thrombosis (n = 6)	In-stent restenosis (n = 10)	Control patients (n = 10)	Healthy subjects (n = 10)
Baseline	1.7 ± 0.4	2.5 ± 0.3	2.4 ± 0.2	2.8 ± 0.5
Substance P (pmol/min)				
4	8.6 ± 0.5	9.6 ± 1.1	9.7 ± 1.2	7.9 ± 1.0
8	9.7 ± 0.7	10.6 ± 1.1	11.0 ± 1.1	9.7 ± 1.3
16	11.6 ± 1.3*	11.3 ± 1.4*	13.2 ± 1.6*	12.4 ± 1.9*
Acetylcholine (μg/min)				
5	7.7 ± 1.8	5.7 ± 1.2	6.2 ± 0.9	6.2 ± 1.3
10	9.7 ± 2.3	6.3 ± 1.4	6.9 ± 1.0	7.3 ± 1.1
20	14.4 ± 3.1 <sup>†</sup>	9.2 ± 2.0 <sup>‡</sup>	9.0 ± 1.5 <sup>‡</sup>	9.4 ± 2.1 <sup>‡</sup>
Sodium nitroprusside (μg/min)				
2	9.7 ± 2.9	7.3 ± 1.0	7.3 ± 0.6	8.4 ± 0.9
4	11.6 ± 2.7	11.1 ± 1.2	10.1 ± 0.9	10.9 ± 1.3
8	13.3 ± 2.6 <sup>‡</sup>	13.4 ± 1.4*	12.6 ± 1.4*	14.4 ± 2.1*

ANOVA for dose response.

\*  $P < 0.0001$ .

<sup>†</sup>  $P < 0.001$ .

<sup>‡</sup>  $P < 0.05$ .

on the platelet-rich plasma, 30–40 min after blood sampling, using a standard optical technique (Chronolog Ca560 aggregometer; Labmedics, Stockport, UK) as described previously

[10], using the following agonists: adenosine diphosphate (ADP, 0.5–10 μmol/l), the thromboxane A2 analogue U46619 (0.5–6 μmol/l), thrombin (200–1000 mU/l) and collagen (1–5 μg/ml). Responses were for 5 min after addition of agonists, and the peak response recorded and adjusted for platelet count ( $250 \times 10^9/l$ ). Platelet count and hematocrit were measured using an automated Coulter counter (Act. 8 Coulter Counter; Beckman-Coulter, High Wycombe, UK).

Venous blood (12 ml) were collected in potassium EDTA and trisodium citrate (Monovette®) tubes and screened for anti-thrombin, protein S and protein C deficiencies, factor V (Leiden) and prothrombin A20210G genotypes and anti-phospholipid antibodies.

### 1.6. Study design

Subjects attended following a 4-h fast and then rested recumbent throughout the study. Strain gauges and cuffs were applied, and the brachial artery of the non-dominant arm was cannulated with a 27-standard wire gauge steel needle (Cooper's Needle Works, Birmingham, UK) under 1% lidocaine (Xylocaine; Astra Pharmaceuticals) local anesthesia and attached to a 16-gauge epidural catheter (Portex). Patency was maintained by infusion of saline via a MS2000 syringe infusion pump (Graesby Medical, Watford, UK). Venous cannulae (17-gauge) were inserted into large

Table 3  
Plasma t-PA and PAI-1 concentrations and release during substance P infusion

	Substance P (pmol/min)	Acute stent thrombosis (n = 6)		In-stent restenosis (n = 10)		Control patients (n = 10)		Healthy subjects (n = 10)	
		Infused arm	Non-infused arm	Infused arm	Non-infused arm	Infused arm	Non-infused arm	Infused arm	Non-infused arm
t-PA antigen (ng/ml)	Baseline	4.9 ± 0.9	4.8 ± 0.9	4.3 ± 0.7	4.1 ± 0.6	4.7 ± 0.3	4.9 ± 0.5	5.1 ± 0.4	5.4 ± 0.5
	4	7.2 ± 1.1	5.2 ± 1.2	5.4 ± 0.8	4.2 ± 0.6	5.8 ± 0.5	4.7 ± 0.4	6.1 ± 0.6	5.4 ± 0.5
	8	7.9 ± 1.1	4.9 ± 1.2	6.0 ± 0.7	4.7 ± 0.6	6.5 ± 0.6	4.7 ± 0.4	7.0 ± 0.8	5.5 ± 0.5
	16	10.3 ± 0.8*	5.6 ± 1.3	8.8 ± 1.5*	4.7 ± 0.6	7.6 ± 0.8 <sup>†</sup>	4.9 ± 0.4	8.5 ± 1.3	7.6 ± 1.5
Estimated t-PA antigen release (ng/100 ml/min)	Baseline	0.1 ± 0.5		0.2 ± 0.4		−0.4 ± 0.4		−0.5 ± 0.4	
	4	11.2 ± 4.1		6.8 ± 2.5		6.9 ± 2.8		3.1 ± 1.3	
	8	18.7 ± 7.7		6.9 ± 2.5		12.2 ± 3.3		7.8 ± 3.3	
	16	34.4 ± 10.1*		25.3 ± 7.5 <sup>‡</sup>		19.6 ± 3.3 <sup>‡</sup>		5.9 ± 2.1*	
Net t-PA antigen release (AUC)		47.1 ± 13.9		26.4 ± 6.6		28.6 ± 8.0		13.6 ± 4.1	
t-PA activity (IU/ml)	Baseline	2.1 ± 0.5	1.8 ± 0.4	2.0 ± 0.3	2.1 ± 0.5	1.2 ± 0.2	1.2 ± 0.2	0.8 ± 0.2	1.0 ± 0.2
	4	5.3 ± 1.0	2.2 ± 0.4	4.6 ± 0.8	2.5 ± 0.6	2.4 ± 0.5	1.29 ± 0.2	2.3 ± 0.5	1.0 ± 0.2
	8	6.7 ± 1.1	2.1 ± 0.4	4.7 ± 0.9	2.7 ± 0.6	3.0 ± 0.6	1.88 ± 0.3	3.8 ± 1.2	1.4 ± 0.3
	16	7.6 ± 1.1 <sup>†</sup>	3.8 ± 0.8	7.0 ± 0.9 <sup>†</sup>	3.6 ± 0.9	5.5 ± 1.0 <sup>†</sup>	2.04 ± 0.4	5.9 ± 1.7	1.7 ± 0.4
Estimated t-PA activity release (IU/100 ml/min)	Baseline	0.1 ± 0.2		−0.1 ± 0.4		0.0 ± 0.1		−0.3 ± 0.1	
	4	16.8 ± 4.6		11.1 ± 3.6		6.6 ± 2.8		5.6 ± 1.8	
	8	27.9 ± 5.9		11.3 ± 3.8		6.1 ± 2.3		16.1 ± 8.5	
	16	25.5 ± 5.5 <sup>†</sup>		18.3 ± 4.2 <sup>†</sup>		25.8 ± 5.3 <sup>‡</sup>		31.5 ± 13.6*	
Net t-PA activity release (AUC)		57.5 ± 10.9		31.5 ± 8.2		25.6 ± 7.7		37.0 ± 16.7	
PAI-1 antigen (ng/ml)	Baseline	33 ± 4	36 ± 4	28 ± 7	30 ± 7	43 ± 8	43 ± 7	36 ± 5	36 ± 5
	16	28 ± 2	33 ± 4	32 ± 7	33 ± 7	42 ± 7	46 ± 8	36 ± 5	34 ± 5

Mean ± S.E.M. ANOVA for dose response.

\*  $P < 0.05$ .

<sup>†</sup>  $P < 0.01$ .

<sup>‡</sup>  $P < 0.001$ .

subcutaneous veins of the antecubital fossae of both arms. After 30 min equilibration with saline infusion, intra-arterial substance P was administered at 4, 8, 16 pmol/min, acetylcholine at 5, 10, 20  $\mu\text{g}/\text{min}$  and sodium nitroprusside at 2, 4, 8  $\mu\text{g}/\text{min}$  for 10 min at each dose [8,11]. The drugs were separated by 20 min of saline infusion and administered in a randomized order. All infusions were given at a constant infusion rate of 1 ml/min. Venous samples were taken at baseline and during infusion of each substance P dose but not during sodium nitroprusside or acetylcholine infusion since they do not affect plasma t-PA or PAI-1 concentrations in this forearm model [8,10,12].

### 1.7. Data analysis and statistics

Plethysmographic and aggregometry data were extracted from the Chart data files and forearm blood flow was calculated for individual venous occlusion cuff inflations by use of a template spreadsheet (Excel 97, Microsoft). Estimated net release of t-PA antigen and activity was defined previously [8] as the product of the infused forearm plasma flow (based on the mean hematocrit and the infused forearm blood flow) and the concentration difference between the infused and noninfused arms. Data was examined, where appropriate, by analysis of variance (ANOVA) with repeated measures and two-tailed paired Student's *t*-test using Statview (SAS Institute). All results are expressed as mean  $\pm$  S.E.M. Statistical significance was taken at the 5% level.

## 2. Results

The baseline characteristics of all subjects are shown in Table 1. The patient groups are matched for age, smoking history, diabetes mellitus, hypertension and severity of coronary artery disease although there appeared to be a greater proportion of female subjects in the acute stent thrombosis group. No significant abnormalities were demonstrated in the thrombophilia screen in all study subjects.

### 2.1. Endothelium-dependent vasomotion

There were no significant changes in heart rate, blood pressure and non-infused forearm blood flow during drug infusion in all studies. Forearm blood flow increased in a dose-dependent manner during substance P, acetylcholine and sodium nitroprusside infusions ( $P < 0.05$ , ANOVA) (Table 2). However, there were no significant differences between the four groups.

### 2.2. Endogenous fibrinolysis

Compared to the non-infused arm, substance P caused dose-dependent increases in plasma t-PA antigen and

activity concentrations in the infused arm in all subjects ( $P < 0.05$ , Table 3), but this increase was not different between the groups. Patients with acute stent thrombosis had an apparently higher t-PA antigen release but this did not achieve statistical significance nor was this seen with t-PA activity. Post-hoc analysis demonstrated a significant reduction of t-PA antigen and activity release in current smokers compared to non-smokers ( $P < 0.05$ , Fig. 1).

### 2.3. Platelet aggregation

All patients were on aspirin therapy and had reduced platelet aggregation in response to collagen ( $P < 0.01$ ) compared to healthy volunteers (Fig. 2). There was no significant difference in platelet aggregation to thrombin, collagen or U46619 between the patient groups (Fig. 2).

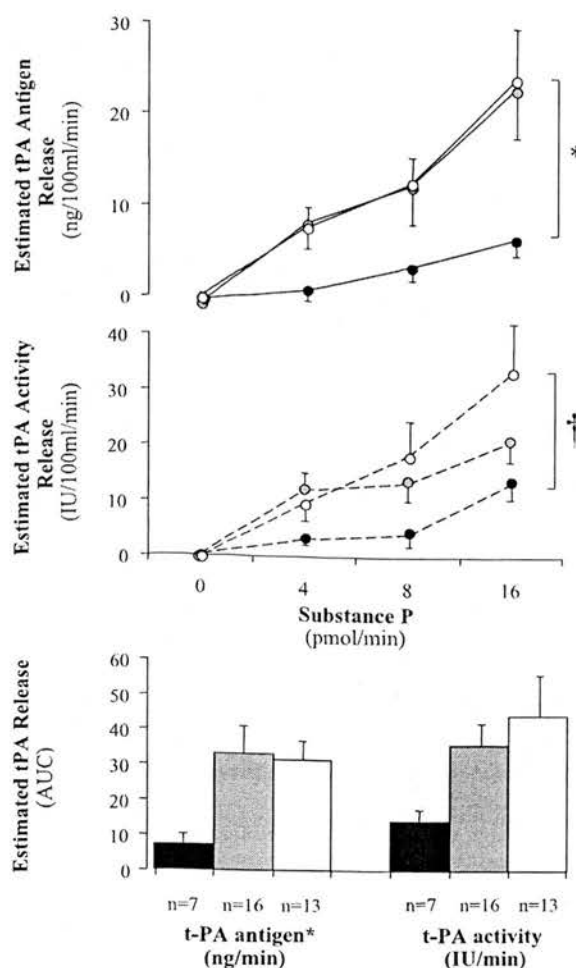


Fig. 1. Estimated net release of t-PA antigen and activity in current smokers (black), ex-smokers (grey) and non-smokers (white). Lower panel represents area under the curve (AUC) for the response. ANOVA for smoking status \* $P < 0.01$ , <sup>†</sup> $P < 0.05$ .

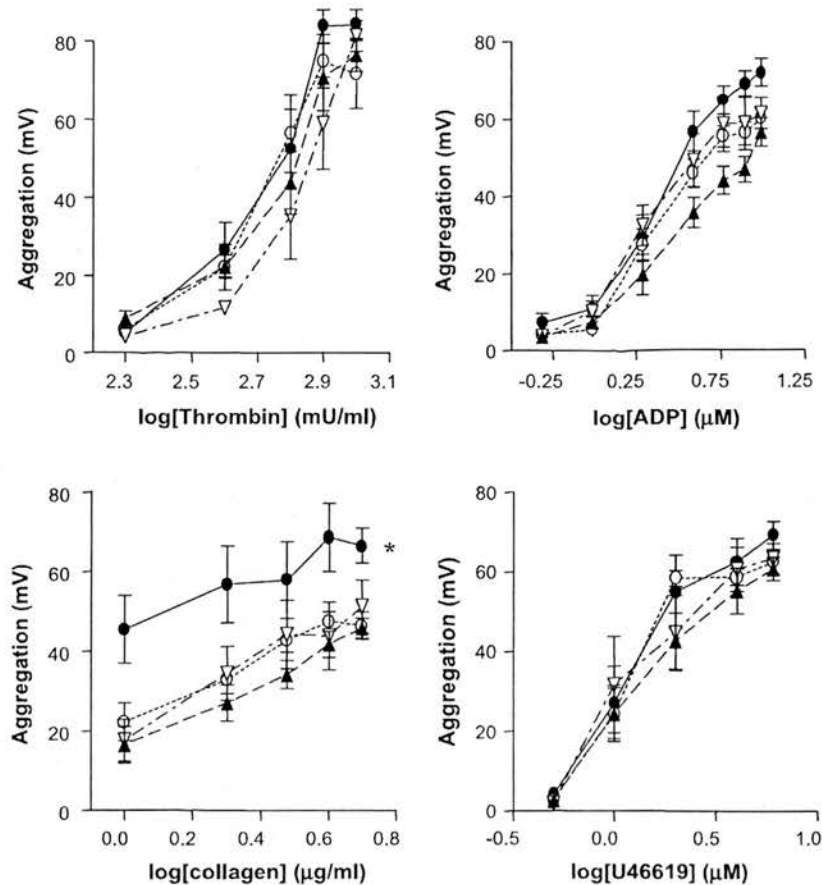


Fig. 2. Platelet aggregation in response to thrombin, collagen, ADP and U46619 in patients with acute stent thrombosis (open inverted triangles), in-stent restenosis (closed triangles) and uncomplicated PCI with stent implantation (open circles), and healthy matched controls (closed circles).  $P < 0.001$  for each dose response. \* $P < 0.01$  controls vs. patients.

However, platelet aggregation was increased in patients with acute stent thrombosis in response to ADP compared to in-stent restenosis ( $P < 0.001$ ), although this was not significant when compared to the control group ( $P = 0.19$ ).

### 3. Discussion

We have assessed three critical aspects of vascular function in patients who have undergone PCI. We have found no evidence to indicate that endothelial vasomotor or fibrinolytic function plays a major role in the pathogenesis of acute stent thrombosis or in-stent restenosis. Moreover, in vitro platelet function appeared to be normal with little evidence of alterations in platelet sensitivity to a number of agonists. In contrast, we have been able to confirm our previous findings [9,13] of the marked inhibition of acute t-PA release in current smokers. We conclude that endothelial and platelet function do not appear to be major determinants of acute stent thrombosis or in-stent restenosis.

#### 3.1. Endothelial vasomotor function

The endothelium plays a critical role in the regulation of vasomotor tone [14,15] and its injury or dysfunction is an important contributing factor in atherothrombosis. There is progressive impairment of endothelium-dependent vasodilatation with the development of coronary atherosclerosis [16] and its associated risk factors, such as hypercholesterolemia [17], smoking [9] and diabetes mellitus [18]. In this study, we assessed forearm blood flow following intra-arterial infusions of the endothelium-dependent vasodilators, substance P and acetylcholine, and the endothelium-independent vasodilator, sodium nitroprusside. We found no differences in the forearm blood flow responses either between the patient groups or comparing the patient and healthy control groups. Given the absence of risk factors and clinically evident disease, one would have anticipated greater endothelium-dependent vasodilatation in the healthy control subjects. However, ageing has a marked effect on the regulation of basal [19] and stimulated [20] endothelium-dependent vascular tone and we studied a predominantly elderly population with a mean age of 62 years. Moreover,



we recognise that subjects who are apparently healthy in this age group commonly have subclinical atherosclerosis that cannot be excluded in the absence of invasive investigation such as coronary angiography.

### 3.2. Endogenous fibrinolysis

The regulated release of endothelial t-PA is an important mechanism in the defence against intravascular thrombosis especially in the coronary circulation [21]. Several investigators have suggested that impaired endogenous fibrinolysis is associated with restenosis after PCI [22,23], and in particular, a rise in plasma PAI-1 antigen concentrations [24]. In the present study, we were unable to find any evidence of impaired endogenous fibrinolysis in our patients with no apparent differences in plasma PAI-1 antigen concentrations or acute endothelial t-PA release. However, when stratifying the subjects according to smoking habit, post-hoc analysis indicated that acute t-PA release was markedly impaired in smokers compared to non-smokers. This confirms our previous findings of impaired t-PA release in smokers [9,13] and gives support to our conclusions that, rather than a lack of power, there appears to be no major impairment of endogenous fibrinolysis in patients with acute stent thrombosis or in-stent restenosis.

### 3.3. Platelet function

Platelet adherence to the arterial wall occurs within minutes of arterial injury. Pathological studies [25] in both porcine coronary arteries and in human saphenous vein grafts have shown that the earliest vascular response to stent implantation is extensive platelet deposition. However, despite important reductions in periprocedural events [26,27], the glycoprotein IIb/IIIa receptor antagonist abciximab does not reduce in-stent restenosis [28]. Consistent with this observation, platelet aggregation in response to thrombin, ADP, the thromboxane A<sub>2</sub> analogue U46619 or collagen was unaffected in our study patient populations. In keeping with long-term aspirin use, patients in all three groups demonstrated reduced collagen-dependent platelet aggregation in comparison with healthy subjects. Again, this suggests that, rather than a lack of power, there appears to be no major difference in platelet aggregation in patients with acute stent thrombosis or in-stent restenosis. Although assessment of platelet function should ideally be performed after aspirin has been withheld for at least 1 week, aspirin use was unavoidable in our patient population.

### 3.4. Study limitations

There are several potential limitations to our clinical study. First, complications from stent implantation may arise due to procedural difficulties or suboptimal stent deployment. However, we were careful to exclude such patients with overt technical problems during the PCI procedure.

Second, acute stent thrombosis is a fortunately rare complication of PCI but this makes recruitment of such patients problematic. This is reflected in the modest number of patients with acute stent thrombosis in our study and means that we lack sufficient power to address the influence of all the individual variables associated with thrombosis, particularly given the higher proportion of female patients in this group. The relatively low number of patients and subjects in the whole study may also raise the potential possibility of a type II error. Third, the assessment in our study is performed at least 6 months following PCI and stent implantation in order to exclude the development of late restenosis in the control patient group. Whilst this avoids the influence of acute confounding factors, we may have missed a transient impairment in endothelial or platelet function. It is therefore possible that changes in platelet behaviour, fibrinolysis or vascular function may occur in the acute phase of stent-thrombosis and this study does not definitely exclude that changes in these factors contribute to the development of acute stent thrombosis. Finally, we used a clinical diagnosis of restenosis and cannot exclude a degree of subclinical angiographic restenosis in the control patient group.

In conclusion, our study suggests that endothelial dysfunction, platelet aggregation and endogenous fibrinolysis do not appear to play a major role in the pathogenesis of acute stent thrombosis or in-stent restenosis.

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## **PUBLICATION 19**

ORIGINAL ARTICLE

## Tissue plasminogen activator genetic polymorphisms do not influence tissue plasminogen activator release in patients with coronary heart disease

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**Summary.** *Objectives:* To determine if polymorphisms of the tissue plasminogen activator (*t-PA*) gene influence acute endogenous *t-PA* release in patients with coronary heart disease (CHD). *Methods:* Forearm blood flow and plasma *t-PA* concentrations were measured in response to intra-brachial infusion of substance P and sodium nitroprusside in 96 patients with stable CHD. Genotyping was performed using a *Taqman* polymerase chain reaction assay specifically designed to detect the polymorphisms of interest: (i) *Alu*-repeat insertion/deletion sequence; (ii) C → T substitution in an upstream enhancer region (–7351 C/T); (iii) T → C in exon 6 (20 099 T/C); and (iv) T → A (27 445 T/A) in intron 10. *Results:* Substance P and sodium nitroprusside caused dose-dependent increases in forearm blood flow in all patients ( $P < 0.001$  for all) that were independent of the four genetic polymorphisms. Similarly, there were no differences in basal plasma *t-PA* antigen concentrations or net *t-PA* release between genotypes. Compared to non-smokers, smokers exhibited impaired substance P-induced vasodilatation ( $P < 0.001$ ) and *t-PA* release ( $P = 0.05$ ). *Conclusions:* Despite confirming our previous findings in cigarette smokers, we have found no effect of polymorphisms of the *t-PA* gene on two complementary aspects of endothelial function. We conclude that genetic variation of the *t-PA* locus is unlikely to have a major influence on acute *t-PA* release in subjects with established CHD.

**Keywords:** coronary heart disease, endothelial function, fibrinolysis, genetic polymorphisms.

### Introduction

Coronary heart disease (CHD) and its risk factors, such as cigarette smoking, hyperlipidemia and hypertension, are associated with impaired endothelium-dependent vasorelaxation [1–3] and tissue plasminogen activator (*t-PA*) release [4–6]. These aspects of endothelial function are important as plasma fibrinolytic variables and endothelium-dependent vasodilatation independently predict future cardiovascular risk [7,8].

Genetic factors influence plasma concentrations of a number of hemostatic and fibrinolytic factors including *t-PA*, plasminogen activator inhibitor type 1 (PAI-1) and fibrinogen [9]. A common intronic polymorphism of the *t-PA* gene is generated by the insertion of a 311 base pair *Alu*-repeat sequence [10]. Although not a consistent finding in all populations [11], there is an independent association between the insertion (II) genotype and the future risk of myocardial infarction (MI): a relative risk of 2.24 in comparison with the deletion (DD) genotype [12]. This *Alu* intronic sequence is now known to be in linkage disequilibrium (LD) with three single nucleotide polymorphisms associated with *t-PA* release, within the upstream enhancer region (7351 C → T), in exon 6 (20 099 T → C) and within intron 10 (27 445 T → A) [13]. The C → T substitution within the enhancer region disrupts a binding site for the Sp1 transcription factor and appears to be functionally important [13,14] with TT homozygotes having an increased risk of both MI [15] and lacunar stroke [16] compared to CC homozygotes.

Genetic differences in *t-PA* release may alter the fibrinolytic balance predisposing to local thrombus formation and an increased likelihood of adverse athero-thrombotic events. Jern and colleagues previously demonstrated a link between *t-PA* release and both the *Alu* insertion/deletion and –7351 C/T enhancer polymorphisms in healthy volunteers [13,17]. However the functional importance of these genetic variants in patients with established CHD is unknown. We hypothesized that polymorphisms of the *t-PA* gene may influence endothelial vasomotor and fibrinolytic function in patients with stable CHD.

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## Methods

### Subjects

We recruited patients with CHD confirmed by angiography (defined as > 70% luminal stenosis of at least one major epicardial coronary vessel) or a history of Q-wave MI. All patients had stable anginal symptoms and had not undergone coronary revascularization within the preceding 3 months. Exclusion criteria were significant cardiac failure, renal impairment, systolic blood pressure < 100 or > 190 mmHg, impaired fasting glucose (> 7 mmol L<sup>-1</sup>) or prior diagnosis of diabetes mellitus. Self-reported smoking status was confirmed using plasma cotinine levels [18]. All studies were undertaken with the approval of the local Research Ethics Committee, the written informed consent of each subject, and in accordance with the Declaration of Helsinki.

### Venous sampling and assays

Venous blood was collected into tubes containing acidified buffered citrate (for t-PA), trisodium citrate (for PAI-1 and cotinine), potassium ethylene diamine tetraacetic acid (for DNA extraction) and serum gel tubes (for C-reactive protein; CRP). Platelet-free plasma, serum and whole blood (DNA) were stored at -80 °C before assay. Plasma t-PA and PAI-1 antigen concentrations were determined using enzyme-linked immunosorbent assays (Coaliza<sup>®</sup>; Chromogenix, Mölndal, Sweden) [5] with intra-assay and inter-assay coefficients of variation of 7.0% and 5.5%, and 4.0% and 7.3% for t-PA and PAI-1 antigen, respectively [19]. All fibrinolytic assays were performed in duplicate and the mean value taken. Serum CRP concentrations were measured using particle-enhanced immunonephelometry (Behring BN II nephelometer, Milton Keynes, UK). Plasma cotinine concentrations were determined using high performance liquid chromatography (ABS Laboratories Ltd, London, UK) [18]. Hematocrit was determined using an automated Coulter counter (Beckman-Coulter ACT.8, High Wycombe, UK). Biochemical assays were undertaken on the fasting venous serum samples by the hospital clinical laboratory facility.

### Genotyping

Genomic DNA was isolated from whole blood. For the -7351 C → T (upstream enhancer), 20 099 T → C (exon 6), and 27 445 T → A (intron 10) polymorphisms genotyping was performed by allele discrimination analysis using the 5' nuclease assay. Oligonucleotide primers and fluorescently labeled probes were obtained from Applied Biosystems (Applied Biosystems, Foster City, CA, USA) on the basis of previously published DNA and primer sequences [13,15]. In brief, polymorphic target sequences were amplified by polymerase chain reaction (PCR) in the presence of two fluorogenic probes specific for each allele. Probes hybridize to their target during the annealing step and the reporter dye is cleaved by the 5' nuclease activity of *Taq* polymerase. Real time quantitative PCR was performed in

an ABI PRISM<sup>®</sup> 7900HT Sequence Detector System (Applied Biosystems). Thermal cycling conditions were two initial holds (50 °C for 2 min and 95 °C for 10 min) followed by a 40-cycle two-step program (95 °C for 15 s and 60 °C for 1 min) utilizing a standardized operating protocol (WTCRF Genetics Core, Edinburgh UK; <http://www.wterf.ed.ac.uk>; accessed 27 July 2006). The fluorescent contribution of each dye was determined directly after the completion of PCR.

For the *Alu*-intronic polymorphism, the insertion specific sequence was submitted to the Applied Biosystems Assay-by-design service. Gene expression assays consisted of unlabeled PCR primers and TaqMan<sup>®</sup> MGB probes (FAM dye labeled). Forward and reverse primers were 5'-CGTAACAGGACAG-CTCAGTT-3 and 5'-CCCTAGGAGAACTTCTCTTTA-ACTTTGTT-3, respectively. The insertion specific probe was labeled in its 5' end with FAM and in its 3' end with a NFQ (non-fluorescent quencher): FAM-ACCCTATGAGATTAGAACAC-NFQ. Real time quantitative PCR was performed with a ABI PRISM<sup>®</sup> 7900HT Sequence Detector using the standardized operating protocol described (WTCRF Genetics Core; <http://www.wterf.ed.ac.uk>; accessed 27 July 2006). *Alu*-insertion PCR products were normalized to threshold values against an endogenous control (single copy RNase P gene). Each sample was run in triplicate with both the gene of interest and the RNase P control. Genotype for the *Alu*-intronic polymorphism was calculated from differences in the threshold cycle between the RNase P and *Alu*-insertion sequence.

A confirmatory assay of *Alu* Ins/Del genotypes utilized PCR amplification of the Ins fragment with primers designed to flank the region of interest (PLAT.1 5' to 3' GTGAAAAGC-AAGGTCTACCAG and PLAT.2 5' to 3' GACACCGAG-TTCATCTTGAC). PCR was performed on a DNA engine tetrad from MJ Research (Bio-Rad Laboratories, Inc, Hercules, CA, USA). Thermal cycling conditions were 94 °C for 2 min followed by 35 cycles (30 s at 94 °C for and 30 s at 60 °C and 30 s at 72 °C) with a final extension step of 10 min at 72 °C. A 10-μL sample of this reaction was analyzed on a 2.5% agarose gel and following electrophoresis DNA was visualized with ethidium bromide and UV transillumination. Amplification of the products produces a 570 bp fragment from chromosomes with the Ins allele and a 260-bp fragment from those without (Del). All genotyping was performed by an individual without information on case status or vascular study data.

### Drugs

Substance P (2, 4 and 8 pmol min<sup>-1</sup>; Clinalfa AG, Switzerland) and sodium nitroprusside (2, 4 and 8 μg min<sup>-1</sup>; David Bull Laboratories, Warwick, UK) were administered following dissolution in 0.9% saline. All solutions were freshly prepared on the day of study.

### Forearm study protocol

Subjects were requested to abstain from alcohol for 24 h, and food, caffeine-containing drinks and tobacco for at least

4 h before each study. Cardioactive medications were withheld on the morning of each study visit. All studies were conducted in a quiet temperature controlled room maintained at 22–25 °C. Patients rested recumbent, strain gauges and cuffs were applied. A 17-G venous cannula was inserted into the antecubital vein of each arm and the brachial artery of the non-dominant arm was cannulated with a 27-SWG needle (Cooper's Needle Works Ltd, Birmingham, UK).

Forearm blood flow was measured in both forearms by venous occlusion plethysmography as previously described [5,20]. Following a 30-min saline infusion to allow equilibration and recording of resting blood flow, intra-arterial substance P at 2, 4 and 8 pmol min<sup>-1</sup> (endothelium-dependent vasodilator releasing t-PA) and sodium nitroprusside at 2, 4 and 8 µg min<sup>-1</sup> (endothelium independent vasodilator which does not release t-PA) [20] were each administered for 6 min in a randomized order with a 15 min saline washout period between each agent. Forearm blood flow was measured at baseline and with each dose of vasodilator. Venous blood sampling for determining plasma t-PA and PAI-1 antigen concentrations was undertaken at baseline and following the recording of blood flow response to each dose of substance P. Blood pressure and heart rate were measured throughout each study using a semi automated non invasive sphygmomanometer (Omron 705 IT; Omron Healthcare UK, Milton Keynes, UK).

#### Statistical analysis

Plethysmographic data were extracted from Chart data files and forearm blood flow calculated for individual venous occlusion cuff inflations by use of a template spreadsheet (Excel 2002®; Microsoft™, Seattle, WA, USA). The last five flow recordings in each 3-min measurement period were calculated and averaged for each arm. Estimated net release of t-PA antigen was defined [5,20] as the product of the infused forearm plasma flow (based on the hematocrit and the infused forearm blood flow), and the concentration difference between the infused and non-infused forearms. Area under the curves (AUCs) for the dose-response of t-PA release were calculated using the trapezoid rule.

Allele frequencies were estimated by gene counting. Chi-squared test was used to evaluate deviations of genotype distributions from the Hardy-Weinberg equilibrium. The LD coefficient D' was calculated as previously described [21]. Baseline characteristics of the study population are presented as frequencies or mean values and standard error of mean. Differences between groups were compared by Student's *t*-test, chi-squared test, and, where appropriate, by analysis of variance (ANOVA) with repeated measures. As CRP values were not normally distributed they were log transformed prior to analysis. All statistical calculations were undertaken using GRAPHPAD PRISM (GraphPad Software, San Diego, CA, USA). Statistical significance was assigned at the 5% level.

**Table 1** Allele frequencies and pairwise linkage disequilibrium (LD) coefficient between polymorphisms at the tissue plasminogen activator (t-PA) locus; (chi-squared, degrees of freedom), *P* < 0.01 for all

		Allelic frequency	Alu	-7351	20 099	27 445
Alu	Ins → Del	0.65/0.35		0.63 (67.4; 4)	0.83 (107.8; 4)	0.60 (76.7; 4)
-7351	C → T	0.69/0.31			0.85 (93.7; 4)	0.68 (82.3; 4)
20 099	T → C	0.63/0.37				0.66 (98.5; 4)
27 445	T → A	0.73/0.27				

#### Results

A total of 96 patients were recruited with a cardiovascular risk factor profile in keeping with their diagnosis of CHD and most were prescribed secondary preventative medications. The genotype distributions for the *Alu* insertion/deletion (II 46%, ID 36% and DD 18%), -7351 C/T enhancer (CC 54%, CT 32%, and TT 14%), 20 099 T/C exon 6 (TT 42%, CT 43%, and CC 15%) and 27 445 T/A intron 10 (TT 56%, TA 34% and AA 10%) were in keeping with the Hardy-Weinberg equilibrium. There was significant LD between the four polymorphisms (Table 1) with allelic frequencies being similar to published studies of European Caucasian subjects [13,17]. There was complete agreement between the two PCR methods in the determination of *Alu* insertion/deletion genotype. Three subjects could be genotyped only for the *Alu* insertion/deletion variant.

Biometric variables were similar between genotypes (Tables 2 and 3; data on file, University of Edinburgh, Edinburgh, UK) although there were significantly fewer smokers in the -7351 heterozygote group (*P* = 0.01, chi-squared).

#### Hemodynamics and forearm blood flow

Resting heart rate, blood pressure and non-infused forearm blood flow were similar in each group and these did not change significantly during infusion of either vasodilator (Tables 2–4; data on file).

Substance P and sodium nitroprusside increased the infused forearm blood flow in all subjects (*P* < 0.001, ANOVA). None of the genetic polymorphisms appeared to influence the endothelium-dependent or -independent blood flow responses (Fig. 1; data on file).

#### Plasma fibrinolytic variables

Baseline plasma t-PA and PAI-1 antigen concentrations were similar for each genotype (Table 4; data on file). Substance P caused a dose-dependent increase in plasma t-PA antigen within the infused arm in all three groups (*P* < 0.01, ANOVA) whilst plasma PAI-1 antigen concentrations were unchanged (data on file). There were no differences in substance P-induced t-PA release between any of the genetic polymorphisms (Fig. 2; data on file). Similarly, the AUC for net t-PA release over the



**Table 2** Clinical characteristics of subjects according to *Alu* insertion/deletion genotype (*n* = 96)

	Genotype			<i>P</i> <sup>†</sup>
	II ( <i>n</i> = 44)	ID ( <i>n</i> = 35)	DD ( <i>n</i> = 17)	
Age, years	59 ± 1	60 ± 1	59 ± 2	0.88
Gender, male/female	33/11	32/3	14/3	0.16
BMI, kg m <sup>-2</sup>	29 ± 1	28 ± 1	30 ± 1	0.27
Systolic BP, mmHg	136 ± 3	128 ± 4	132 ± 4	0.23
Diastolic BP, mmHg	79 ± 2	75 ± 2	73 ± 2	0.30
Pulse, bpm	57 ± 1	58 ± 2	57 ± 3	0.96
Current smoker (%)	30	23	47	0.42
Co-morbidity (%)				
Previous MI	43	46	24	0.28
Hypertension	50	49	47	0.98
FHx CHD	52	37	29	0.74
Prior hyperlipidemia	93	94	94	1.00
Extent of coronary disease, (%) <sup>*</sup>				
1 vessel	41	51	41	0.33
2 vessels	27	20	24	0.86
3 vessels	30	17	35	0.43
Medical therapy, (%)				
Aspirin	100	100	94	0.10
Statin therapy	91	97	94	0.52
ACE inhibitor, ARB	27	34	29	1.00
Urea, mmol L <sup>-1</sup>	5.4 ± 0.2	5.6 ± 0.3	5.7 ± 0.4	0.70
Creatinine, µmol L <sup>-1</sup>	93.1 ± 2.5	94.6 ± 2.1	91.3 ± 2.6	0.72
Glucose, mmol L <sup>-1</sup>	5.6 ± 0.1	5.7 ± 0.2	6.0 ± 0.2	0.24
Total chol, mmol L <sup>-1</sup>	4.5 ± 0.2	4.4 ± 0.2	4.4 ± 0.2	0.89
LDL chol, mmol L <sup>-1</sup>	2.6 ± 0.1	2.5 ± 0.2	2.2 ± 0.2	0.28
Triglycerides, mmol L <sup>-1</sup>	1.7 ± 0.2	1.7 ± 0.2	2.3 ± 0.3	0.09
CRP, mg L <sup>-1</sup>	1.87 ± 0.18	1.92 ± 0.35	1.90 ± 0.41	0.97

Values are mean ± SEM.

<sup>\*</sup>Angiographic data unavailable on five subjects. <sup>†</sup>*P* = ANOVA or chi-squared for difference between genotypes. BMI, body mass index; bpm, beats per minute; CHD, coronary heart disease; MI, myocardial infarction; FHx, family history; chol, cholesterol; ACE, angiotensin converting enzyme; ARB, angiotensin receptor blocker; CRP, C-reactive protein.

30-min period of substance P infusion was similar between genotypes (data on file).

Smoking status was confirmed by plasma cotinine concentration (median 307.8 vs. 0.6 ng mL<sup>-1</sup> in smokers and non-smokers respectively). Subgroup analysis revealed an interaction between smoking status and vascular function. Consistent with our previous findings, cigarette smokers had impaired forearm blood flow (FBF) responses (*P* < 0.001, ANOVA) and net t-PA release (*P* = 0.05, ANOVA; Fig. 3) compared to non-smokers. In light of the uneven distribution of smokers across -7351 genotypes and the interaction between smoking and t-PA release, we performed a *post hoc* analysis of t-PA release according to smoking status. However, we found no difference in the AUC for net t-PA release between CC, CT and TT -7351 genotypes in either smokers (111 [48–174]; 167 [94–240]; 193 [5–382]; *P* = 0.41 ANOVA) or non-smokers (187 [114–260]; 160 [100–220]; 188 [50–325]; *P* = 0.83) respectively (mean [95% confidence intervals]).

**Table 3** Clinical characteristics of subjects according to -7351 C/T genotype (*n* = 93)

	Genotype			<i>P</i> <sup>†</sup>
	CC ( <i>n</i> = 50)	CT ( <i>n</i> = 30)	TT ( <i>n</i> = 13)	
Age, years	59 ± 1	59 ± 1	61 ± 3	0.82
Gender, male/female	38/12	27/3	11/2	0.28
BMI, kg m <sup>-2</sup>	29 ± 1	28 ± 1	29 ± 1	0.47
Systolic BP, mmHg	134 ± 3	131 ± 4	123 ± 6	0.27
Diastolic BP, mmHg	79 ± 2	75 ± 2	70 ± 3	0.07
Pulse, bpm	57 ± 1	57 ± 2	54 ± 3	0.57
Current smoker (%)	46	13	38	0.01
Co-morbidity (%)				
Previous MI	44	43	38	0.89
Hypertension	46	40	62	0.43
FHx CHD	32	37	31	0.23
Prior hyperlipidemia	92	93	92	0.98
Extent of coronary disease, (%) <sup>*</sup>				
1 vessel	40	50	38	0.64
2 vessels	24	23	23	0.99
3 vessels	30	20	38	0.42
Medical therapy, (%)				
Aspirin	100	97	100	0.35
Statin therapy	88	100	92	0.14
ACE inhibitor, ARB	28	23	54	0.98
Urea, mmol L <sup>-1</sup>	5.2 ± 0.2	5.7 ± 0.2	6.1 ± 0.5	0.09
Creatinine, µmol L <sup>-1</sup>	93.3 ± 2.2	92.6 ± 2.1	92.3 ± 4.0	0.96
Glucose, mmol L <sup>-1</sup>	5.6 ± 0.1	5.6 ± 0.2	6.0 ± 0.3	0.26
Total chol, mmol L <sup>-1</sup>	4.6 ± 0.2	4.4 ± 0.2	4.2 ± 0.3	0.37
LDL chol, mmol L <sup>-1</sup>	2.7 ± 0.1	2.6 ± 0.1	2.0 ± 0.3	0.08
Triglycerides, mmol L <sup>-1</sup>	1.8 ± 0.2	1.6 ± 0.1	2.4 ± 0.3	0.08
CRP, mg L <sup>-1</sup>	2.02 ± 0.29	1.5 ± 0.3	2.3 ± 0.7	0.49

Values are mean ± SEM.

<sup>\*</sup>Angiographic data unavailable on five subjects. <sup>†</sup>*P* = ANOVA or chi-squared for difference between genotypes. BMI, body mass index; bpm, beats per minute; CHD, coronary heart disease; MI, myocardial infarction; FHx, family history; chol, cholesterol; ACE, angiotensin converting enzyme; ARB, angiotensin receptor blocker; CRP, C-reactive protein.

## Discussion

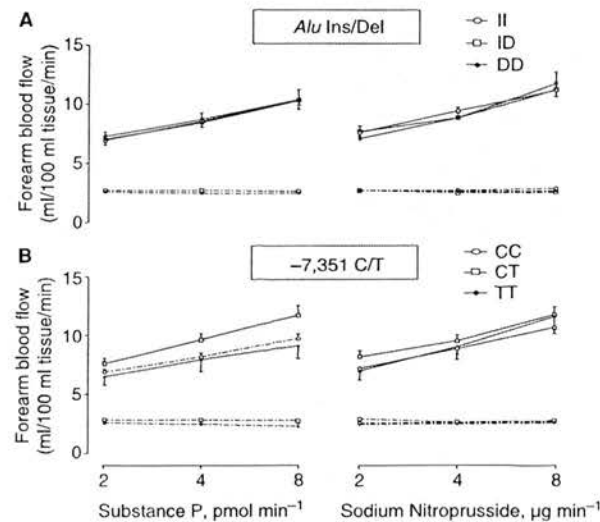
This is the first study to assess directly the role of *t-PA* genetic polymorphisms in determining acute t-PA release in patients with CHD. Using a validated and reproducible technique [22], we have found no difference in the basal concentration, or net release of, plasma t-PA antigen between genotypes. These important findings suggest that, in patients with established coronary atherosclerosis, variation at the *t-PA* gene locus does not substantially influence acute endogenous fibrinolytic capacity.

Tissue plasminogen activator release from endothelial cells is the major arbiter of local intravascular fibrinolysis [23] and will influence vessel patency following acute coronary thrombosis [24]. Inter-individual differences in t-PA release may contribute to a pro-thrombotic phenotype and determine the sequelae of acute MI. Indeed, clustering of individuals with low rates of t-PA release has been reported in families with a history of thrombosis [25]. Moreover, genetic murine models of t-PA

**Table 4** Effect of *Alu* insertion/deletion and -7351 C/T genotypes on baseline forearm blood flow, tissue type-plasminogen activator (t-PA) and plasminogen activator inhibitor type 1 (PAI-1) antigen concentrations; *P* = ns between genotypes

	<i>Alu</i> Insertion/deletion			-7351 C/T		
	II	ID	DD	CC	CT	TT
Forearm blood flow (mL 100 mL tissue <sup>-1</sup> min <sup>-1</sup> )						
Infused arm	2.7 ± 0.1	2.8 ± 0.2	2.7 ± 0.2	2.6 ± 0.1	2.8 ± 0.2	2.6 ± 0.3
Non-infused arm	2.7 ± 0.2	2.7 ± 0.2	2.5 ± 0.2	2.6 ± 0.1	2.9 ± 0.2	2.4 ± 0.3
t-PA antigen (ng mL <sup>-1</sup> )						
Infused arm	9.1 ± 0.5	10.7 ± 0.8	9.0 ± 0.7	9.4 ± 0.5	9.8 ± 0.8	9.3 ± 1.1
Non-infused arm	9.3 ± 0.5	10.9 ± 0.8	8.8 ± 0.7	9.6 ± 0.5	9.9 ± 0.8	9.3 ± 1.1
PAI-1 antigen (ng mL <sup>-1</sup> )						
Infused arm	43.3 ± 4.2	43.6 ± 3.7	44.1 ± 6.6	44.5 ± 3.5	44.3 ± 4.8	39.8 ± 7.4
Non-infused arm	46.0 ± 4.2	44.0 ± 3.6	43.7 ± 6.5	47.7 ± 3.6	42.4 ± 4.6	40.7 ± 7.2

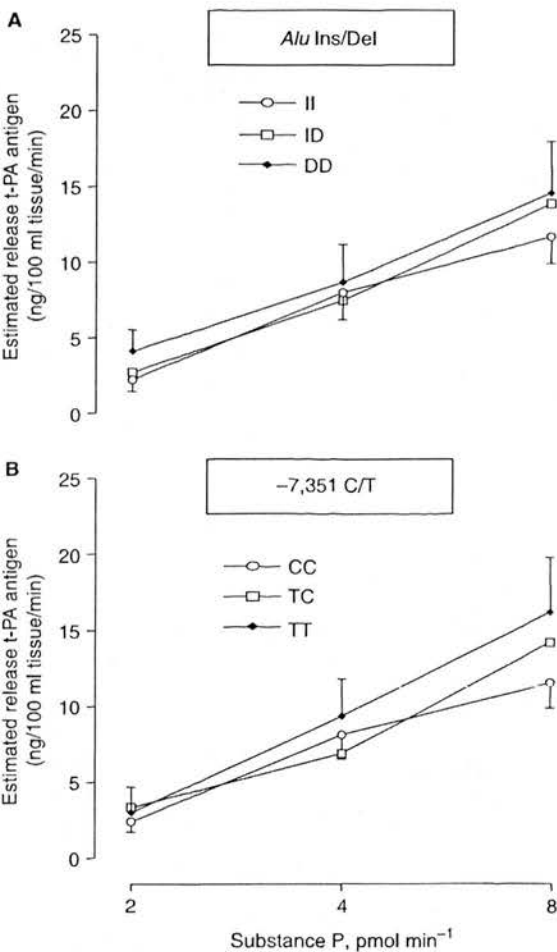
Values are mean ± SEM.



**Fig. 1.** Infused (solid line) and non-infused (dashed line) forearm blood flow during intra-brachial substance P (left) and sodium nitroprusside (right) according to (A) *Alu* insertion/deletion, and (B) -7351 C/T, genotypes. *P* < 0.001, ANOVA, for infused FBF response to both vasodilators.

deficiency show a reduced thrombolytic potential and an increased thrombotic tendency [26]. We therefore wanted to explore the potential genetic influences on t-PA release in at risk patients with CHD.

The t-PA -7351C → T enhancer site polymorphism has been associated with an increased risk of first MI [15] and lacunar stroke [16] although the data on non-lacunar stroke is conflicting [16,27]. The strong linkage between the -7351 C/T enhancer and the *Alu*-repeat polymorphisms may explain the association between the (non-coding) *Alu* insertion/deletion polymorphism and cardiovascular events [12,13]. In healthy volunteers (*n* = 51), both the *Alu* insertion/deletion and -7351 C → T enhancer polymorphisms were associated with significant differences in basal t-PA release with II and CC homozygotes having around 2-fold greater release rates than the DD and TT homozygotes respectively [13,17]. Of the genotypes studied here, the 7351 C → T substitution is thought to result in a functional change occurring within an Sp1 transcription



**Fig. 2.** Estimated net release of tissue plasminogen activator (t-PA) antigen induced by intra-brachial substance P (2, 4 and 8 pmol min<sup>-1</sup>) according to (A) *Alu* insertion/deletion, and (B) -7351 C/T, genotypes. *P* < 0.001, ANOVA, for dose response.

factor binding site which is negatively regulated by binding of the thymidine allele [13]. However despite this difference in basal t-PA release rate, acute t-PA release was similar between all genotypes. Now, in a larger cohort of subjects (*n* = 96) with

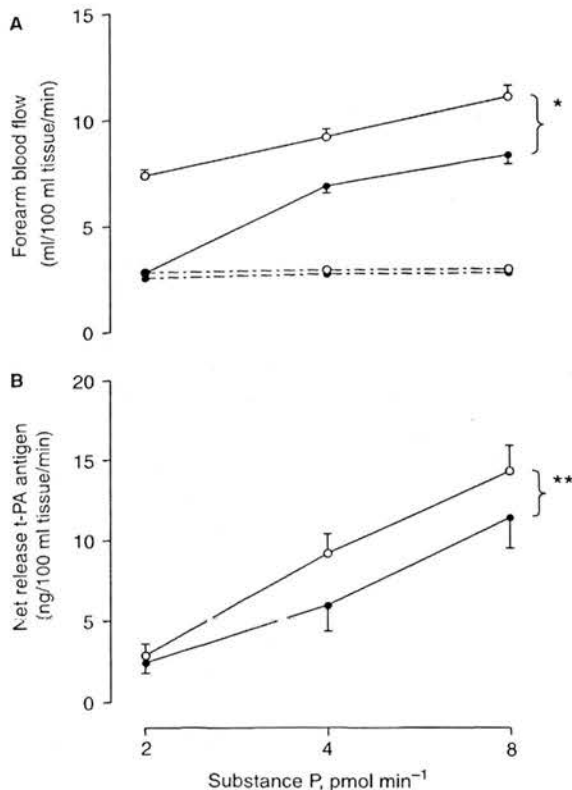


Fig. 3. Infused (solid line) and non-infused (dashed line) forearm blood flow (A), and net t-PA release (B), in response to intra-brachial substance P in smokers (filled circle) and non-smokers (hollow circle). \* $P < 0.001$ , 2-way ANOVA, infused FBF response in smokers vs. non-smokers; \*\* $P = 0.05$ , 2-way ANOVA, net t-PA release in smokers vs. non-smokers.

established CHD, we have shown that there is no significant relationship between basal plasma t-PA concentrations or acute t-PA release, and these genetic polymorphisms. Furthermore, endothelium-dependent vasodilatation, a complementary measure of endothelial function, was similarly unaffected by variation within the *t-PA* gene locus.

Regulatory control of t-PA release involves distinct pathways from those controlling vessel calibre and indeed t-PA release can be induced without changes in forearm blood flow [28]. In view of previous work in healthy volunteers [13,17], we chose to assess whether a number of *t-PA* genetic polymorphisms were associated with changes in vascular function in patients with CHD. If t-PA genotype was important in regulating endogenous t-PA release, certain alleles could favor the continued presence of thrombus following atheromatous plaque rupture. Organization of residual thrombus is known to stimulate further plaque growth and expansion [29], disrupting endothelial integrity that is critical to the regulation of vascular tone and coagulation. Impaired endothelium-dependent vasodilatation is considered a surrogate of endothelial dysfunction and has been widely studied as a bio-marker of atherosclerosis. We therefore hypothesized that *t-PA* genetic polymorphisms might also be associated with changes in endothelium-depend-

ent vasodilatation through pathways linking coagulation, atherogenesis, and endothelial dysfunction. However, we found no evidence to support the contention that t-PA polymorphisms influence endothelial function in patients with CHD. In contrast, cigarette smoking and CHD are associated with endothelial dysfunction as demonstrated by impaired endothelium-dependent vasodilatation [1] and endogenous t-PA release [5,30]. Our study confirms these previous findings in cigarette smokers in a large cohort of patients with established CHD maintained on secondary preventative medications. These consistent findings would appear to reaffirm the validity of our approach and suggest that environmental rather than genetic factors have a more dominant influence on acute endogenous t-PA release.

There are a number of differences between the current study and previous reports demonstrating an interaction between acute t-PA release and polymorphisms within the *t-PA* gene locus. Our population comprised subjects with established CHD recruited within the UK. In contrast, Jern *et al.* [13,17] studied healthy Scandinavian volunteers free of symptomatic atherosclerosis. A recent meta analysis of studies on genetic polymorphisms and CHD risk demonstrated heterogeneity according to the population studied [31]. The *Alu*-repeat polymorphism was associated with an increased incidence of acute MI in Dutch subjects [12] but not in studies from North America [11] or the UK [32]. Our subjects were all of European Caucasian origin, recruited from within the same geographical area, and had similar risk factor profiles. It seems unlikely that biological variability or modulation from environmental factors obscured true differences in t-PA release between genotypes. Although our findings may not be applicable to other racial groups, Rosenbaum and colleagues [33] failed to demonstrate differences in acute endogenous t-PA release between healthy black and white Americans.

#### Study limitations

Although endothelium-dependent blood flow responses are similar in the forearm and coronary circulations [34], we cannot be sure that these polymorphisms of the *t-PA* gene do not influence fibrinolytic responses within the coronary circulation. Indeed, there are limited data to suggest that, although not apparent in the forearm circulation, angiotensin-converting enzyme gene insertion/deletion polymorphism can influence acute t-PA release in the coronary circulation [35].

Jern and colleagues previously demonstrated a difference in basal, though not stimulated, t-PA release according to t-PA genotype [13,17]. Whilst we cannot determine basal t-PA release using the venovenous technique described, we have found no relationship between t-PA genotype and stimulated t-PA release. Calculation of net t-PA release provides an accurate assessment of stimulated t-PA release with good reproducibility [19], and basal release contributes only a small proportion of the overall venous plasma t-PA

concentration. In this study we did not find any difference in acute t-PA release in response to substance P between t-PA genotypes. However, our study protocol cannot exclude the possibility that these polymorphisms might alter protein transcription and endothelial storage, or the very early (< 6 min) release of, t-PA in response to substance P infusion [36].

In our cohort, there was an uneven distribution of smokers between t-PA -7351 CC, CT and TT groups. Although we found no difference in net t-PA release between these three groups when analyzed according to smoking status, we accept that the relationship between cigarette smoking and impaired t-PA release may have obscured an association between t-PA genotype and fibrinolytic function with the *post hoc* analysis limited by the number of smokers and non-smokers within each group.

## Conclusion

Whilst we have confirmed previous findings showing that cigarette smokers have impaired endothelial vasomotor and fibrinolytic function, we have found no major effect of genetic variation within the *t-PA* gene locus on either plasma t-PA concentrations or acute t-PA release in subjects with stable CHD.

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## Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

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**PUBLICATION 20**

# Endothelial dysfunction in patients with recent myocardial infarction and hyperhomocysteinaemia: effects of vitamin supplementation

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## A B S T R A C T

Hyperhomocysteinaemia is a prothrombotic condition that may cause oxidative endothelial injury and impair endogenous fibrinolysis. Vitamin supplementation enhances endothelial function in hyperhomocysteinaemic patients, but responses in patients with co-existing coronary artery disease have been variable. It is also unknown whether hyperhomocysteinaemia is associated with reduced fibrinolytic responses in patients with coronary artery disease. The study aims were to test the hypothesis that patients with recent myocardial infarction and hyperhomocysteinaemia have impaired endothelium-dependent vasomotion and fibrinolysis that is rectified by vitamin supplementation. From a cohort of 120 patients admitted with acute myocardial infarction, 18 patients were recruited from the upper ( $n=9$ ) and lower ( $n=9$ ) plasma homocysteine quartiles into a randomized double-blind placebo-controlled crossover trial. Following a 4-week course of placebo or folate/cyanocobalamin/pyridoxine supplements, FBF (forearm blood flow) was measured using venous occlusion plethysmography during intra-arterial substance P (4–16 pmol/min), acetylcholine (5–20  $\mu$ g/min) and sodium nitroprusside (2–8  $\mu$ g/min) infusions. All vasodilators caused dose-dependent increases in infused FBF ( $P<0.05$ ). Patients in the upper homocysteine quartile ( $16.8 \pm 2.9$  compared with  $7.9 \pm 0.7$   $\mu$ mol/l;  $P=0.003$ ) had reduced vasodilatation to acetylcholine ( $P=0.01$ ) and substance P ( $P<0.05$ ), but not sodium nitroprusside. There were no differences in substance P-induced tissue plasminogen activator release. Vitamin supplementation increased serum folate and vitamin B<sub>12</sub> concentrations ( $P<0.05$ ), but did not significantly lower homocysteine, or affect FBF or fibrinolytic responses. In patients with recent myocardial infarction, hyperhomocysteinaemia is associated with impaired endothelium-dependent vasodilatation, but no alteration in the acute fibrinolytic capacity. This endothelial vasomotor dysfunction is unaltered by vitamin supplementation.

## INTRODUCTION

Several prospective and case-control studies have shown that elevated plasma homocysteine concentrations are an independent risk factor for the development of athero-

thrombotic vascular disease as well as a prognostic marker in ischaemic heart disease [1–3]. Plasma homocysteine concentrations are consistently higher in patients with premature peripheral and cerebrovascular diseases [4], and almost a third of patients with premature coronary

**Key words:** endothelial dysfunction, fibrinolysis, homocysteine, myocardial infarction, vasodilatation, vitamin.

**Abbreviations:** CI, confidence interval; FBF, forearm blood flow; NS, not significant; PAI-1, plasminogen activator inhibitor type 1; t-PA, tissue plasminogen activator.

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artery disease are found to have hyperhomocysteinaemia [1]. In addition, apparently healthy men with plasma homocysteine concentrations 12% above the upper limit of normal have a 3-fold increased risk of acute myocardial infarction [2], and in patients with ischaemic heart disease there is an increased mortality associated with plasma concentrations greater than  $9 \mu\text{mol/l}$  [5].

The vascular endothelium plays a central role in the control of blood flow, haemostasis and endogenous fibrinolysis, and endothelial dysfunction independently predicts cardiovascular events [6,7]. Although the mechanism of vascular damage is unclear, homocysteine may promote atherogenesis through oxidative endothelial injury that is mediated by cytotoxic reactive oxygen species [8–10]. Indeed, acute and chronic hyperhomocysteinaemia are associated with impaired endothelium-dependent flow-mediated dilatation of the brachial artery [10–12].

Hyperhomocysteinaemia is a prothrombotic state [3]. We [13] and others [14] have shown previously that hyperhomocysteinaemia induced by oral methionine loading is associated with alterations in endogenous fibrinolysis in healthy subjects and patients with premature vascular disease. However, the influence of chronic hyperhomocysteinaemia on the acute fibrinolytic capacity is unknown and is the subject of debate [15]. Interestingly, there is an association between plasma homocysteine and t-PA (tissue plasminogen activator) antigen concentrations in stroke patients [16].

Vitamin supplementation with folate, vitamin B<sub>6</sub> and vitamin B<sub>12</sub> is safe and may reduce plasma homocysteine concentrations [17]. Although endothelial function is enhanced following treatment with folate in patients with hyperhomocysteinaemia [18,19] and hypercholesterolaemia [20], the response in patients with coronary artery disease has been variable [21–25]. Furthermore, it is unknown whether elevated plasma homocysteine concentrations are associated with reduced resistance vessel vasomotor responses in patients with established coronary artery disease.

The aim of the present study was to test the hypotheses that, in patients with recent myocardial infarction, elevated plasma homocysteine concentrations are associated with impaired endothelium-dependent vasodilatation and endogenous fibrinolytic capacity, and that vitamin supplementation (with folate, vitamin B<sub>6</sub> and vitamin B<sub>12</sub>) would both lower plasma homocysteine and restore endothelial function.

## METHODS

### Subject recruitment

One hundred and twenty patients admitted with an acute myocardial infarction were recruited into the trial. Myocardial infarction was defined as typical ischaemic cardiac

pain associated with elevation of cardiac markers (greater than twice the upper limit of normal) and electrocardiographic evidence of myocardial ischaemia. Exclusion criteria were atrial fibrillation on warfarin therapy, impaired renal function (serum creatinine  $>120 \mu\text{mol/l}$ ), diabetes mellitus, requirement for folate supplementation or pernicious anaemia. The written informed consent of each subject was obtained before entry into the study. All studies were undertaken with the approval of the local Research Ethics Committee and in accordance with the Declaration of Helsinki (1996).

### Study design

Fasting plasma homocysteine concentrations were determined in all patients on days 5–7 following acute myocardial infarction [26]. From the upper and lower plasma homocysteine concentration quartiles, nine patients in each quartile were recruited into a randomized double-blind balanced-block placebo-controlled crossover trial at least 4 months after the index event. All patients received two separate 4-week courses of oral sucrose placebo or vitamin supplementation (5 mg of folate/100  $\mu\text{g}$  of cyanocobalamin/10 mg of pyridoxine), and attended at the end of each 4-week treatment period. On each study day, medications were withheld and subjects attended after a 4-h fast and rested recumbent in a quiet temperature-controlled room maintained at 22–25°C. Strain gauges and cuffs were applied, and the brachial artery of the non-dominant arm was cannulated. After 30 min equilibration with saline infusion, intra-arterial substance P (4–16 pmol/min), acetylcholine (5–20  $\mu\text{g/min}$ ) and sodium nitroprusside (2–8  $\mu\text{g/min}$ ) were administered in a randomized order for 6–10 min at each dose and separated by 20 min washout periods [27–29]. Venous samples were taken at baseline and during infusion of each substance P dose for determination of t-PA and PAI-1 (plasminogen activator inhibitor type 1). Venous sampling was not performed during sodium nitroprusside or acetylcholine infusion, since they do not affect t-PA or PAI-1 release in this forearm model [13,27,30].

### Intra-arterial administration and drugs

The brachial artery of the non-dominant arm was cannulated with a 27-standard wire gauge steel needle (Cooper's Needle Works, Birmingham, U.K.) under 1% lidocaine (Xylocaine; Astra Pharmaceuticals, Kings Langley, Herts., U.K.) local anaesthesia and attached to a 16-gauge epidural catheter (Portex, Hythe, Kent, U.K.). Patency was maintained by infusion of saline (0.9% NaCl) via a MS2000 syringe infusion pump (Graseby Medical, Watford, Herts., U.K.). The total rate of intra-arterial infusions was maintained constant throughout all studies at 1 ml/min. Pharmaceutical grade substance P (Clinalfa, Läufelfingen, Switzerland), acetylcholine (Cibavision Ophthalmics, Southampton, U.K.) and sodium nitroprusside (David Bull Laboratories, Warwick,

U.K.) were administered following dissolution in saline. All solutions were freshly prepared on the day of study.

### Haemodynamic measurements

Blood flow was measured in both forearms by venous occlusion plethysmography using mercury-in-silastic strain gauges as described previously [27,28]. During measurement periods, the hands were excluded from the circulation by rapid inflation of the wrist cuffs to 220 mmHg using E20 rapid cuff inflators (DE Hokanson, Bellevue, WA, U.S.A.). Upper arm cuffs were inflated intermittently to 40 mmHg for 8 s in every 10 s to achieve venous occlusion and obtain plethysmographic recordings. Blood pressure was monitored in the non-infused arm at intervals throughout each study with a semi-automated non-invasive oscillometric sphygmomanometer (Takeda UA 751; Takeda Medical, Tokyo, Japan).

### Venous sampling and assays

Venous cannulae (17-gauge) were inserted into large subcutaneous veins of the antecubital fossae of both arms. Venous blood was withdrawn simultaneously from each arm and collected into acidified buffered citrate (Biopool® Stabilyte™, Umeå, Sweden) for t-PA assays and trisodium citrate and lithium heparin tubes (Monovette®, Sarstedt, Nümbrecht, Germany) for PAI-1 and homocysteine assays respectively. Samples were kept on ice before being centrifuged at 2000 g. Platelet-free plasma was decanted and stored at -80°C before assay. Plasma t-PA and PAI-1 antigens were determined as described previously [27] using ELISA (Coaliza t-PA and Coaliza PAI-1; Chromogenix, Mölndal, Sweden), and plasma t-PA activity was determined using a photometric method (Coatest t-PA; Chromogenix). Plasma homocysteine (Axis® Homocysteine EIA; Axis-Shield, Oslo, Norway) and serum folate and vitamin B<sub>12</sub> concentrations (Bayer Immuno 1® automated immunoassay analyser, Bayer, Leverkusen, Germany) were determined using enzyme immunoassay.

### Data analysis and statistics

Estimated net release of t-PA antigen and activity were defined previously [27] as the product of the infused forearm plasma flow [based on the mean haematocrit (Hct) and the infused FBF (forearm blood flow)] and the concentration difference between the infused ([t-PA]<sub>inf</sub>) and non-infused ([t-PA]<sub>Noninf</sub>) arms using the formula:

Estimated net t-PA release

$$= \text{FBF} \times (1 - \text{Hct}) \times ([\text{t-PA}]_{\text{inf}} - [\text{t-PA}]_{\text{Noninf}})$$

Data were examined, where appropriate, by ANOVA with repeated measures and two-tailed paired Student's *t* test (SAS Institute). All results are expressed as means (S.E.M.) or means [95% CI (confidence interval)]. Statistical significance was taken at the 5% level.

**Table 1 Patient characteristics**

Values are means (S.E.M.), or numbers (percentage). ACE, angiotensin-converting enzyme.

	Patients in upper quartile	Patients in lower quartile
<i>n</i>	9	9
Age (years)	54 (2)	57 (5)
Sex (male/female)	9/0	8/1
Mean arterial pressure (mmHg)	88 (5)	86 (2)
Heart rate (beats/min)	59 (2)	54 (2)
Type of myocardial infarction		
Anterior ( <i>n</i> )	2 (22%)	3 (33%)
Inferior ( <i>n</i> )	6 (67%)	3 (33%)
Other ( <i>n</i> )	1 (11%)	3 (33%)
Peak creatine kinase (units/l)	790 (230)	1900 (550)
Received thrombolysis ( <i>n</i> )	6 (67%)	6 (67%)
Medication		
Antiplatelet therapy ( <i>n</i> )	9 (100%)	9 (100%)
β-Adrenoceptor antagonists ( <i>n</i> )	8 (89%)	6 (67%)
ACE inhibitors ( <i>n</i> )	5 (56%)	5 (56%)
HMG CoA reductase inhibitors ( <i>n</i> )	8 (89%)	7 (77%)
Risk factors		
Smokers ( <i>n</i> )	6 (67%)	3 (33%)
History of hypertension ( <i>n</i> )	3 (33%)	2 (22%)
Hypercholesterolaemia ( <i>n</i> )	8 (89%)	7 (77%)
Diabetes mellitus ( <i>n</i> )	0 (0%)	0 (0%)

## RESULTS

### Baseline and biochemical characteristics

From the upper and lower plasma homocysteine quartiles, 18 patients (nine patients in each quartile) were recruited into the randomized controlled trial. Apart from plasma homocysteine concentrations ( $P < 0.01$ , as determined by Student's *t* test), which were significantly different by design, there were no significant differences between the baseline clinical characteristics or medical therapies in the two groups of patients (Tables 1 and 2).

All subjects tolerated placebo and vitamin supplementation and no side effects were reported or noted. Serum folate and vitamin B<sub>12</sub> concentrations were increased following vitamin supplementation compared with placebo in both patient groups ( $P < 0.001$  and  $P < 0.05$  respectively, as determined by Student's *t* test; Table 2). Plasma homocysteine concentrations appeared to be reduced by  $\approx 16\%$  with active treatment in hyperhomocysteinaemic patients, but this was not statistically significant ( $P = 0.3$ , as determined by Student's *t* test; Table 2). Vitamin supplementation had no significant effects on heart rate, blood pressure or basal FBF in either patient group.

**Table 2** Plasma homocysteine, serum folate and vitamin B<sub>12</sub> concentrations following vitamin supplementation

Values are means (S.E.M.). \* $P < 0.01$  compared with lower quartile, as determined by Student's  $t$  test; † $P < 0.05$  compared with placebo, as determined by Student's  $t$  test.

	Patients in the upper quartile		Patients in the lower quartile	
	Placebo	Treatment	Placebo	Treatment
Plasma homocysteine ( $\mu\text{mol/l}$ )	16.8 (2.8)*	14.2 (1.1)*	7.9 (0.7)	7.8 (0.7)
Serum folate ( $\mu\text{g/l}$ )†	8.6 (1.8)	17.7 (1.6)†	9.0 (1.2)	19.9 (0.1)†
Serum vitamin B <sub>12</sub> (ng/l)	462 (136)	580 (149)†	380 (39)	431 (48)†

† Upper limit of serum folate assay was 20  $\mu\text{g/l}$ ; 13 patients had concentrations above this after supplementation and were taken as 20  $\mu\text{g/l}$ .

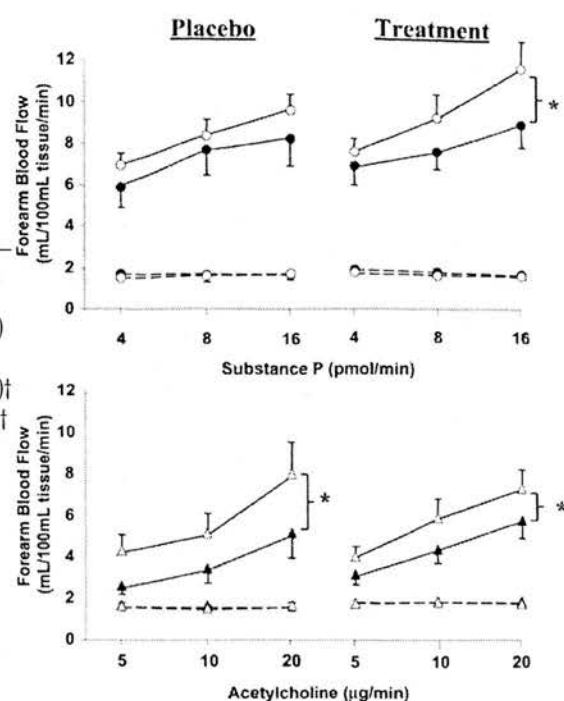
### Endothelium-dependent vasomotion

Substance P, acetylcholine and sodium nitroprusside caused dose-dependent increases in blood flow in the infused forearm in both patient groups on each study day ( $P < 0.05$ , as determined by ANOVA; Figure 1). In comparison with patients in the lower quartile, hyperhomocysteinaemic patients had significantly reduced FBF responses to acetylcholine and substance P ( $P = 0.01$  and  $P < 0.05$  respectively, as determined by ANOVA; Figure 1), but there were no significant differences in the blood flow responses to sodium nitroprusside [ $P = \text{NS}$  (not significant), as determined by ANOVA].

Neither endothelium-dependent nor endothelium-independent vasodilatation were significantly influenced by vitamin treatment in either patient group ( $P = \text{NS}$  for all, as determined by ANOVA). For the hyperhomocysteinaemic group, the mean difference for the response to vitamin treatment at the peak dose was  $1.1 \text{ ml} \cdot 100 \text{ ml}^{-1} \cdot \text{min}^{-1}$  (95% CI,  $-0.8$  to  $+2.9$ ) for substance P,  $0.3 \text{ ml} \cdot 100 \text{ ml}^{-1} \cdot \text{min}^{-1}$  (95% CI,  $-1.3$  to  $+1.8$ ) for acetylcholine, and  $-0.3 \text{ ml} \cdot 100 \text{ ml}^{-1} \cdot \text{min}^{-1}$  (95% CI,  $-2.8$  to  $+2.1$ ) for sodium nitroprusside. For the lower quartile group, the mean difference for the response to vitamin treatment at the peak dose was  $2.0 \text{ ml} \cdot 100 \text{ ml}^{-1} \cdot \text{min}^{-1}$  (95% CI,  $-0.6$  to  $+4.6$ ) for substance P,  $-0.8 \text{ ml} \cdot 100 \text{ ml}^{-1} \cdot \text{min}^{-1}$  (95% CI,  $-2.9$  to  $+1.2$ ) for acetylcholine, and  $1.7 \text{ ml} \cdot 100 \text{ ml}^{-1} \cdot \text{min}^{-1}$  (95% CI,  $-2.8$  to  $+6.2$ ) for sodium nitroprusside.

### Fibrinolytic responses

Baseline plasma t-PA and PAI-1 antigen and t-PA activity concentrations were similar in both groups (Table 3). Substance P caused dose-dependent increases in plasma t-PA antigen and activity concentrations in the infused forearm of all patients ( $P < 0.05$  for both, as determined by ANOVA; Table 3). The responses were similar in both patient groups and were not influenced by vitamin



**Figure 1** Infused (solid line) and non-infused (dashed line) FBF responses to incremental doses of substance P (upper panel) and acetylcholine (lower panel) in patients in upper (closed symbols) and lower quartiles (open symbols) following placebo (left panel) and vitamin supplementation (right panel)

$P < 0.05$  for all infused arm responses, as determined by ANOVA. \* $P < 0.05$  compared with lower quartile, as determined by ANOVA.

supplementation ( $P = \text{NS}$ , as determined by ANOVA). The mean difference of t-PA antigen release for the response to vitamin supplementation at the peak substance P dose was  $-2.7 \text{ ng} \cdot 100 \text{ ml}^{-1} \cdot \text{min}^{-1}$  (95% CI,  $-15.5$  to  $+10.2$ ;  $P = \text{NS}$ , as determined by Student's  $t$  test) in the upper quartile group and  $-7.4 \text{ ng} \cdot 100 \text{ ml}^{-1} \cdot \text{min}^{-1}$  (95% CI,  $-24.3$  to  $+9.5$ ;  $P = \text{NS}$ , as determined by Student's  $t$ ) in the lower quartile group. Plasma PAI-1 concentrations were unaffected by substance P infusion or vitamin treatment ( $P = \text{NS}$ , as determined by Student's  $t$  test).

### DISCUSSION

In the present study, we have demonstrated that, in patients with a recent myocardial infarction, elevated plasma homocysteine concentrations are associated with impaired endothelium-dependent vasodilatation without affecting acute endogenous t-PA release. However, vitamin supplementation failed to significantly reduce plasma homocysteine concentrations or improve endothelial vasomotor function.



**Table 3 Plasma t-PA and PAI-I concentrations and release during substance P infusion**Values are means (S.E.M.). \* $P < 0.05$  for all responses during substance P infusion, as determined by ANOVA. AUC, area under curve; IU, international units.

	Substance P (pmol/min)	Patients in the upper quartile				Patients in the lower quartile			
		Placebo		Treatment		Placebo		Treatment	
		Infused arm	Non-infused arm	Infused arm	Non-infused arm	Infused arm	Non-infused arm	Infused arm	Non-infused arm
t-PA antigen (ng/ml)	Baseline	7.7 (1.2)	8.6 (1.4)	7.5 (1.0)	8.2 (1.4)	6.6 (1.2)	6.5 (1.3)	7.2 (1.3)	7.6 (1.3)
	4	9.8 (1.5)	8.3 (1.3)	9.2 (1.0)	7.7 (1.3)	7.6 (0.9)	7.0 (1.1)	8.1 (1.4)	7.6 (1.4)
	8	9.4 (1.5)	8.1 (1.1)	10.3 (1.1)	7.8 (1.0)	9.3 (1.4)	7.1 (1.2)	8.9 (1.5)	7.6 (1.5)
	16	12.7 (2.4)*	7.8 (0.9)	11.1 (1.4)*	7.6 (1.1)	10.6 (1.2)*	6.7 (1.3)	10.5 (1.6)	7.9 (1.4)
Estimated t-PA antigen release (ng · 100 ml <sup>-1</sup> · min <sup>-1</sup> )	Baseline	— 1.1 (0.7)		— 1.2 (1.1)		0.4 (0.7)		— 0.3 (0.4)	
	4	4.0 (3.2)		5.4 (3.4)		3.4 (2.2)		1.4 (1.5)	
	8	4.7 (4.1)		11.1 (4.2)		9.8 (3.7)		5.9 (2.4)	
	16	21.6 (6.7)*		18.9 (5.2)*		24.3 (8.4)*		15.6 (3.9)*	
Net t-PA antigen release (AUC)		18.9 (9.3)		25.4 (9.0)		24.2 (8.5)		14.9 (4.2)	
t-PA activity (IU/ml)	Baseline	0.9 (0.2)	0.8 (0.2)	0.5 (0.1)	0.6 (0.1)	1.0 (0.3)	1.0 (0.3)	0.8 (0.2)	0.8 (0.3)
	4	1.7 (0.6)	0.7 (0.2)	1.5 (0.3)	0.8 (0.1)	2.5 (0.8)	1.2 (0.4)	1.5 (0.6)	0.9 (0.2)
	8	1.9 (0.7)	0.8 (0.3)	2.3 (0.4)	0.7 (0.2)	2.8 (0.9)	1.2 (0.4)	2.0 (0.9)	0.9 (0.2)
	16	3.1 (0.8)*	1.0 (0.2)	2.9 (0.5)*	0.8 (0.2)	3.9 (1.1)*	1.5 (0.4)	2.7 (1.0)*	0.9 (0.2)
Estimated t-PA activity release (IU · 100 ml <sup>-1</sup> · min <sup>-1</sup> )	Baseline	0.0 (0.1)		— 0.1 (0.1)		0.0 (0.1)		0.1 (0.2)	
	4	2.5 (1.4)		3.1 (1.3)		6.0 (2.1)		2.2 (1.2)	
	8	3.6 (1.6)		7.0 (1.7)		8.4 (3.6)		4.6 (2.1)	
	16	9.3 (2.9)*		11.7 (3.6)*		16.4 (6.9)*		10.2 (3.4)*	
Net t-PA activity release (AUC)		10.7 (4.2)		15.9 (4.7)		21.6 (8.7)		12.0 (4.9)	
PAI-I antigen (ng/ml)	Baseline	47 (5)	48 (4)	44 (4)	48 (5)	45 (14)	46 (14)	44 (11)	46 (13)
	16	48 (4)	54 (3)	45 (4)	50 (5)	44 (15)	44 (15)	46 (12)	49 (15)

## Endothelium-dependent vasodilatation

Previous studies have shown that homocysteine is associated with endothelial dysfunction. Lentz et al. [9] reported that, in monkeys with diet-induced hyperhomocysteinaemia, endothelium-dependent vasodilatation is impaired in carotid artery rings *in vitro* and hindlimb resistance vessels *in vivo*. Celermajer et al. [11] documented abnormal endothelium-dependent vasodilatation in children with severe hyperhomocysteinaemia due to homozygous homocystinuria. Impaired endothelium-dependent vasodilatation is detected in healthy subjects with acute hyperhomocysteinaemia induced by oral methionine loading [10,31], as well as in patients with chronic hyperhomocysteinaemia who are free from clinical manifestations of atherosclerotic disease [12,18]. In the present study, we have extended these findings to patients with recent myocardial infarction and demonstrated impaired vasomotor responses in those with elevated plasma homocysteine concentrations.

The vascular endothelium plays a critical role in the control of vascular homeostasis by regulating vascular tone, platelet activity, coagulation and fibrinolysis, and endothelial dysfunction is believed to be an early step in the pathogenesis and pathophysiology of atherosclerosis.

Although patients with coronary artery disease typically demonstrate endothelial dysfunction, there is considerable heterogeneity in the magnitude of impairment in individuals with similar risk factor profiles. This is of particular interest because the extent of coronary as well as peripheral endothelial dysfunction independently predicts the long-term risk of acute cardiovascular events, including sudden cardiac death, myocardial infarction and revascularization procedures [6,7]. Recent prospective data have indicated that, in patients with established coronary artery disease, homocysteine is a significant predictor of mortality independent of other traditional risk factors [5,32]. Our present findings therefore support the role of homocysteine as a secondary risk marker, suggesting that this may be mediated through its effects on endothelial function.

## Endogenous fibrinolysis

Although homocysteine impairs endothelial vasomotor function, it does not appear to have a major effect on endothelium-dependent fibrinolytic capacity, as both basal and stimulated release of t-PA or PAI-1 were not significantly different between the two patient groups. Hyperhomocysteinaemia is a prothrombotic condition

and may interfere with the antithrombotic and fibrinolytic mechanisms of the endothelium and alter endothelial protein secretory pathways. Although endothelial cell-associated t-PA activity is reduced in homocysteine-treated cells [33], our present study failed to detect reduced fibrinolytic activity *in vivo*. This is consistent with data indicating that homocysteine might perturb the intrinsic fibrinolytic potential by reducing the functional binding site for t-PA without altering the catalytic capability of t-PA or affecting t-PA synthesis and secretion [33].

### Effects of vitamin supplementation on plasma homocysteine

Previous studies have shown that treatment with folate and B vitamins can lower plasma homocysteine concentrations to a varying degree. The Homocysteine Lowering Trialists' meta-analysis predicted a 20–30% reduction in homocysteine in patients with plasma concentrations above 12  $\mu\text{mol/l}$  taking folate, and a further small additional effect with vitamin B<sub>12</sub> but not B<sub>6</sub> [17]. However, a more modest 11–14% reduction is seen in patients with coronary artery disease who consumed fortified breakfast cereals [34,35]. The limited homocysteine lowering seen in our present study was probably related to the relatively mild hyperhomocysteinaemia and normal folate concentrations in our study population as well as the confounding effects of dietary folate fortification.

### Effects of vitamin supplementation on endothelial responses

We did not detect an improvement in endothelium-dependent vasodilatation or endogenous fibrinolytic capacity following vitamin supplementation in the present study. Although this may not be surprising in the absence of significant homocysteine reduction, Doshi et al. [22] have suggested that the acute effects of folate on endothelial function may occur by a mechanism independent of homocysteine lowering. Moreover, the evidence of the beneficial effects of vitamin supplementation on endothelial function is conflicting. Chambers et al. [21] and Title et al. [24] demonstrated improved endothelial function in patients with coronary artery disease following folate treatment without or with vitamin B<sub>12</sub>. However, our present findings are consistent with those of other investigators who failed to detect improved endothelial function in a similar patient population, in healthy siblings of patients with premature atherothrombotic disease or in patients with renal impairment [23,25,36]. These contradictory findings may be related, in part, to the presence of other cardiovascular risk factors such as hypertension or hypercholesterolaemia, which may contribute to endothelial injury [37,38], but would not be expected to respond to folate or B vitamins. The vascular

endothelium in patients with established coronary artery disease may have also been subjected to chronic injury and would therefore be less responsive to intervention. Furthermore, although the above studies adopted flow-mediated dilatation as a non-invasive method of assessing conduit artery endothelial function, in the present and other studies [28,39], we have focussed on the function of endothelium within resistance vessels. Conduit artery and microvascular endothelial cells have distinct phenotypic differences, and responses to mechanical rather than pharmacological stimuli may also differ, and may contribute to the apparent disparity in the responses.

There is further controversy regarding the effects of vitamin supplementation on cardiovascular outcomes in patients undergoing percutaneous coronary intervention. Contrary to earlier reports that vitamin supplementation may reduce the rate of restenosis and adverse outcomes following coronary artery angioplasty [40,41], Lange et al. [42] have recently demonstrated that folate therapy following coronary stenting may increase the risk of in-stent restenosis. The underlying mechanism for these findings remains uncertain, but it is possible that the proliferative effects of folate may promote the growth of neointimal cells within implanted stents. Therefore more prospective data are needed before any recommendations can be made regarding the use of vitamin supplementation in coronary artery disease.

### Study limitations

There are several potential limitations to our present study. First, we studied peripheral vascular function and thus these findings may not be directly applicable to other vascular beds. However, endothelial dysfunction is often a generalized process, and we have shown previously consistent vasomotor and endogenous fibrinolytic responses between the forearm and coronary circulation [27,39]. Secondly, the failure to improve endothelial function may be related to inadequate treatment duration, although studies have suggested that a 4–6-week treatment with folate can improve endothelial function [20,22]. Thirdly, the size of the study was small and, although it was powered to detect a 15–20% difference in t-PA release or forearm vasodilatation, it is possible that a smaller effect may have been missed. Fourthly, we used a placebo-controlled crossover design and there was the possibility of a carry-over effect of the vitamin therapy on endothelial function during placebo administration, despite a significant difference in the serum concentrations of folate and vitamin B<sub>12</sub>. Finally, we cannot rule out that homocysteine may be a marker of vascular injury rather than a mediator of endothelial dysfunction, although experimental data support the direct role of homocysteine in causing endothelial damage [9,33].

In conclusion, we have demonstrated that endothelium-dependent vasodilatation, but not endogenous

fibrinolysis, is impaired in patients with recent myocardial infarction and elevated plasma homocysteine, and that this endothelial vasomotor dysfunction is not rectified by vitamin supplementation. These results provide further evidence for the role of homocysteine in vascular damage, but do not support the hypothesis that vitamin supplementation improves endothelial function in patients with established coronary artery disease.

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**PUBLICATION 21**



## CARDIOVASCULAR MEDICINE

# Phosphodiesterase type 5 inhibition does not reverse endothelial dysfunction in patients with coronary heart disease

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**Objectives:** To investigate whether sildenafil citrate, a selective phosphodiesterase type 5 inhibitor, may improve endothelial vasomotor and fibrinolytic function in patients with coronary heart disease.

**Design:** Randomised double blind placebo controlled crossover study.

**Patients and methods:** 16 male patients with coronary heart disease and eight matched healthy men received intravenous sildenafil or placebo. Bilateral forearm blood flow and fibrinolytic parameters were measured by venous occlusion plethysmography and blood sampling in response to intrabrachial infusions of acetylcholine, substance P, sodium nitroprusside, and verapamil.

**Main outcome measures:** Forearm blood flow and acute release of tissue plasminogen activator.

**Results:** Mean arterial blood pressure fell during sildenafil infusion from a mean (SEM) of 92 (1) to 82 (1) mm Hg in patients and from 94 (1) to 82 (1) mm Hg in controls ( $p < 0.001$  for both). Sildenafil increased endothelium independent vasodilatation with sodium nitroprusside ( $p < 0.05$ ) but did not alter the blood flow response to acetylcholine or verapamil in patients or controls. Substance P caused a dose dependent increase in plasma tissue plasminogen activator antigen concentrations ( $p < 0.01$ ) that was unaffected by sildenafil in either group.

**Conclusions:** Sildenafil does not improve peripheral endothelium dependent vasomotor or fibrinolytic function in patients with coronary heart disease. Phosphodiesterase type 5 inhibitors are unlikely to reverse the generalised vascular dysfunction seen in patients with coronary heart disease.

The endothelium is important in the regulation of vascular function including local blood flow and endogenous fibrinolysis. Coronary heart disease (CHD) and its risk factors, such as cigarette smoking, hyperlipidaemia, and hypertension,<sup>1-3</sup> are associated with impaired endothelium dependent vasorelaxation and reduced endothelial release of the endogenous fibrinolytic factor tissue plasminogen activator (t-PA).<sup>4-6</sup> These aspects of endothelial function are important, since plasma fibrinolytic variables and endothelium dependent vasodilatation independently predict future cardiovascular risk.<sup>6-7</sup>

Nitric oxide is a key factor linked to the beneficial protective effects of the endothelium and a decrease in nitric oxide bioavailability favours atherogenesis.<sup>8</sup> Nitric oxide exerts many of its biological effects through generation of cyclic guanosine monophosphate (cGMP) after activation of soluble guanylate cyclase. Phosphodiesterase type 5 (PDE5) inactivates cGMP within vascular smooth muscle and thus negatively regulates nitric oxide mediated cellular actions.<sup>9</sup> Recently, highly selective PDE5 inhibitors that prolong the action of cGMP and thereby enhance nitric oxide mediated effects have become available for clinical use.

It has been suggested that the PDE5 inhibitor sildenafil citrate (Viagra; Pfizer) can improve endothelial vasomotor function in the peripheral circulation of healthy cigarette smokers<sup>10</sup> and the coronary circulation of patients with CHD.<sup>11</sup> Although we have previously reported a link between nitric oxide and acute t-PA release,<sup>12</sup> the potential beneficial effects of sildenafil on t-PA and its inhibitor, plasminogen activator inhibitor type 1 (PAI-1), are unknown.

We therefore hypothesised that sildenafil would favourably alter endothelium dependent vasomotor function and acute t-PA release in patients with stable CHD. If so, PDE5 inhibitors may become a useful adjunctive treatment

and confer secondary preventative benefits on patients with CHD.

## METHODS

### Patients

Sixteen male patients with stable CHD and eight age matched healthy control men participated in the study. The investigation was undertaken with the approval of the local research ethics committee, with the written informed consent of each patient, and in accordance with the principles outlined in the Declaration of Helsinki.

A history of CHD was confirmed by angiographic evidence of  $> 50\%$  luminal stenosis of at least one major epicardial coronary vessel or a history of myocardial infarction (confirmed by a serial rise in creatine kinase of twice the upper limit of the normal reference range and the development of pathological Q waves in at least two contiguous leads of the ECG). Nitrate medications were withdrawn for 48 hours before each visit and other medications were withheld on the morning of study. Patient exclusion criteria were significant cardiac failure, renal impairment, systolic blood pressure  $< 100$  or  $> 190$  mm Hg, and diabetes mellitus. Control subjects were healthy normotensive euglycaemic non-smokers without any history of cardiorespiratory or vascular disease and were not taking any regular medications. No participant had received sildenafil or other phosphodiesterase inhibitors before or during participation in this study.

**Abbreviations:** cGMP, cyclic guanosine monophosphate; CHD, coronary heart disease; FBF, forearm blood flow; PAI-1, plasminogen activator inhibitor type 1; PDE5, phosphodiesterase type 5; t-PA, tissue plasminogen activator

## Measurements

Forearm blood flow (FBF) was measured in both forearms by venous occlusion plethysmography with mercury in silastic strain gauges applied to the widest part of the forearm as previously described.<sup>12-14</sup> During measurement periods the hands were excluded from the circulation by rapid inflation of the wrist cuffs to a pressure of 220 mm Hg with E20 rapid cuff inflators (DE Hokanson Inc, Bellevue, Washington, USA). Upper arm cuffs were inflated intermittently to 40 mm Hg for 10 seconds in every 15 seconds to achieve venous occlusion and obtain plethysmographic recordings. Analogue voltage output from an EC-4 strain gauge plethysmograph (DE Hokanson) was processed by an analogue to digital converter and Chart version 5 software (AD Instruments Ltd, Chalgrove, UK). Instruments were calibrated with the internal standard of the plethysmograph. Blood pressure and heart rate were monitored in the non-infused arm by a semiautomated non-invasive sphygmomanometer (Agilent V24; Phillips Medical Systems). Mean arterial pressure was defined as the diastolic pressure plus a third of the pulse pressure.

Plasma t-PA and PAI-1 antigen concentrations were measured as previously described with enzyme linked immunosorbent assays (Coaliza t-PA and PAI-1; Chromogenix AB, Mölndal, Sweden) at baseline, after sildenafil or placebo, and during intra-arterial substance P.<sup>4, 12-14</sup> Haematocrit was determined by an automated Coulter counter (ACT.8; Beckman-Coulter, High Wycombe, UK). Biochemical assays were undertaken on the fasting venous samples by the hospital clinical laboratory facility.

## Study design

Participants were requested to abstain from alcohol for 24 hours and from food, caffeine-containing drinks, and tobacco for at least four hours before each study. All studies were carried out in a quiet temperature controlled room maintained at 22–25°C. Each participant attended at 9 am on two separate occasions at least two weeks apart and received matched placebo and sildenafil in a randomised double blind crossover design.

While participants rested recumbent, strain gauges and cuffs were applied. A 17 gauge venous cannula was inserted into the antecubital vein of each arm and a 23 gauge cannula into the dorsal foot vein for the administration of either intravenous sildenafil or matched placebo. The brachial artery of the non-dominant arm was cannulated with a 27-SWG needle (Cooper's Needle Works Ltd, Birmingham, UK) under local anaesthesia. The intra-arterial infusion rate was maintained constant at 1 ml/min throughout the study with an IVAC syringe pump (Alaris Medical Ltd, Basingstoke, UK).

Saline was infused intra-arterially for the first 20 minutes to allow recording of resting FBF, blood pressure, and heart rate. After this period, sildenafil or matched placebo (Pfizer UK Ltd, Sandwich, Kent, UK) was administered intravenously as a single 26.25 mg bolus over five minutes, then as a continuous infusion of 10 mg/hour to achieve stable plasma concentrations equivalent to the peak concentration of a single 100 mg oral dose (pharmacokinetic data, Pfizer UK Ltd). Twenty minutes after the sildenafil or placebo infusion was started, basal FBF was determined and thereafter acetylcholine (5, 10, and 20 µg/min; Novartis UK Ltd, Farnborough, UK), substance P (2, 4, and 8 pmol/min; Clinalfa AG, Läufelfingen, Switzerland), sodium nitroprusside (2, 4, and 8 µg/min; David Bull Laboratories, Warwick, UK), and verapamil (10, 30, and 100 µg/min; Abbott UK Ltd) were infused intra-arterially for six minutes at each dose. Acetylcholine, substance P, and sodium nitroprusside were given in a random order and separated by 20 minute saline washout periods but, because of its prolonged vasodilator

**Table 1** Baseline characteristics

	Patients	Controls
Age (years)	57 (2)	54 (2)
Body mass index (kg/m <sup>2</sup> )	27 (1)	27 (1)
Co-morbidity		
Myocardial infarction	10	0
Hypertension	12	0
Diabetes mellitus	0	0
Previous hyperlipidaemia	15	0
Smoker/non-smoker	1/15	0/8
Medications		
Aspirin	16	0
β Adrenergic blocker	13	0
Calcium antagonist	3	0
Long acting nitrate/nicorandil	2	0
ACE inhibitor, AT II antagonist	5	0
Lipid lowering agent	16	0
Serum urea (mmol/l)	5.5 (0.3)	5.1 (0.4)
Serum creatinine (µmol/l)	92 (3)	95 (4)
Glucose (mmol/l)	5.6 (0.2)	5.5 (0.3)
Total cholesterol (mmol/l)	4.2 (0.2)	5.5 (0.2)*
HDL cholesterol (mmol/l)	1.0 (0.0)	1.1 (0.1)
Triglycerides (mmol/l)	1.7 (0.2)	1.2 (0.2)
Placebo visit		
Heart rate (beats/min)	55 (1)	61 (3)*
MAP (mm Hg)	95 (2)	95 (4)
FBF (ml/100 ml/min)		
Infused arm	2.5 (0.2)	2.2 (0.2)
Non-infused arm	2.3 (0.2)	2.1 (0.4)
Sildenafil visit		
Heart rate (beats/min)	55 (1)	65 (3)*
MAP (mm Hg)	95 (2)	91 (3)
FBF (ml/100 ml/min)		
Infused arm	2.5 (0.2)	2.6 (0.3)
Non-infused arm	2.5 (0.2)	2.4 (0.3)

Data are mean (SEM) or number.

\**p* < 0.001 unpaired *t* test, patients v controls.

ACE, angiotensin converting enzyme; AT II, angiotensin II type I receptor; FBF, forearm blood flow; HDL, high density lipoprotein; MAP, mean arterial pressure.

action, verapamil was infused last. The order of the infusions was maintained constant for each participant across both visits.

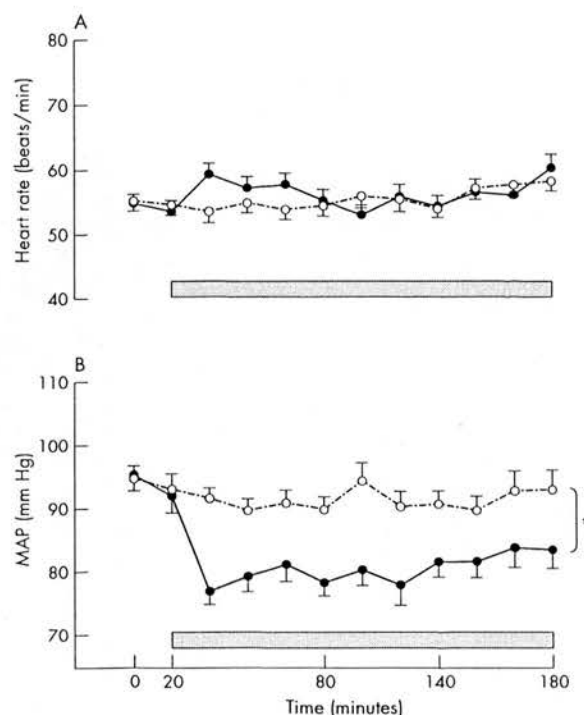
## Statistical analysis

Plethysmographic data were extracted from Chart data files from which the last five linear recording in each measurement period were averaged and FBF was calculated. Estimated net t-PA antigen was defined as the product of the infused forearm plasma flow (based on the haematocrit and the infused FBF) and the concentration difference between the infused ([t-PA]<sub>inf</sub>) and non-infused ([t-PA]<sub>non-inf</sub>) forearms,<sup>12-14</sup> where estimated net t-PA release = FBF × (1 – haematocrit) × ([t-PA]<sub>inf</sub>) – ([t-PA]<sub>non-inf</sub>).

Data were examined, where appropriate, by analysis of variance with repeated measures and two tailed Student's *t* test by GraphPad Prism (GraphPad Software, San Diego, California, USA). All results are expressed as mean (SEM). Significance was assigned at the 5% level. On the basis of a previous study,<sup>15</sup> this study had an 80% power to detect a 23% change in plasma t-PA concentrations and a 22% difference in FBF in patients with CHD between sildenafil and placebo at the 5% level.

## RESULTS

Most patients with CHD had a history of myocardial infarction, hypertension, and hyperlipidaemia (table 1). Reflecting concomitant treatment, mean resting heart rate (55 (1) v 63 (2) beats/min, respectively, *p* < 0.001, unpaired *t* test) and serum total cholesterol concentration (4.2 (0.2) v 5.5 (0.2) mmol/l, *p* < 0.001) were lower in patients with CHD than in controls. Baseline mean arterial pressure,



**Figure 1** (A) Heart rate and (B) mean arterial pressure (MAP) during sildenafil (closed circles, solid line) or placebo (open circles, dashed line) infusion (shaded box) in patients with coronary heart disease (CHD). \* $p < 0.001$  analysis of variance, sildenafil versus matched placebo. Control subject data on file ( $p < 0.001$ , analysis of variance, MAP sildenafil versus matched placebo).

resting heart rate, baseline FBF, or haematocrit did not differ between the two study visits. Infusions were well tolerated and there were no serious adverse events. For technical reasons, one control subject was unable to complete both visits.

### Haemodynamic effects

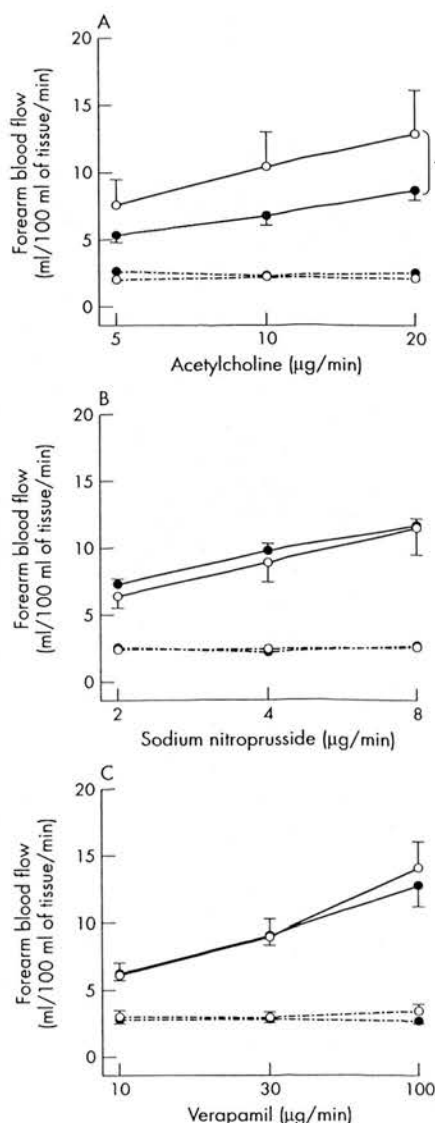
Over the course of the study, the average mean arterial pressure was lower during sildenafil than placebo infusion in patients with CHD (82 (1) v 92 (1) mm Hg,  $p < 0.001$  paired  $t$  test sildenafil versus placebo) (fig 1) and control subjects (82 (1) v 94 (1) mm Hg,  $p < 0.001$  paired  $t$  test). It returned to baseline after discontinuation of infusion (data not shown). Heart rate rose transiently after the sildenafil bolus in both groups (fig 1 and data on file).

### Placebo visit

Acetylcholine caused a dose dependent increase in FBF in both groups, although this rise was significantly less in patients with CHD than in controls ( $p = 0.005$ , analysis of variance) (fig 2). FBF responses did not differ between the two groups during sodium nitroprusside and verapamil infusions (fig 2). There were no significant changes in the non-infused FBF.

### Sildenafil and vascular function

Compared with placebo, administration of sildenafil caused no significant difference in the infused FBF during intra-arterial infusion of acetylcholine (at 20  $\mu\text{g}/\text{min}$ , mean difference 0.1 ml/100 ml/min, 95% confidence interval (CI) -0.2 to 0.4), substance P (at 8 pmol/min, mean difference 0.5 ml/100 ml/min, 95% CI 0.00 to 0.9), or verapamil (at

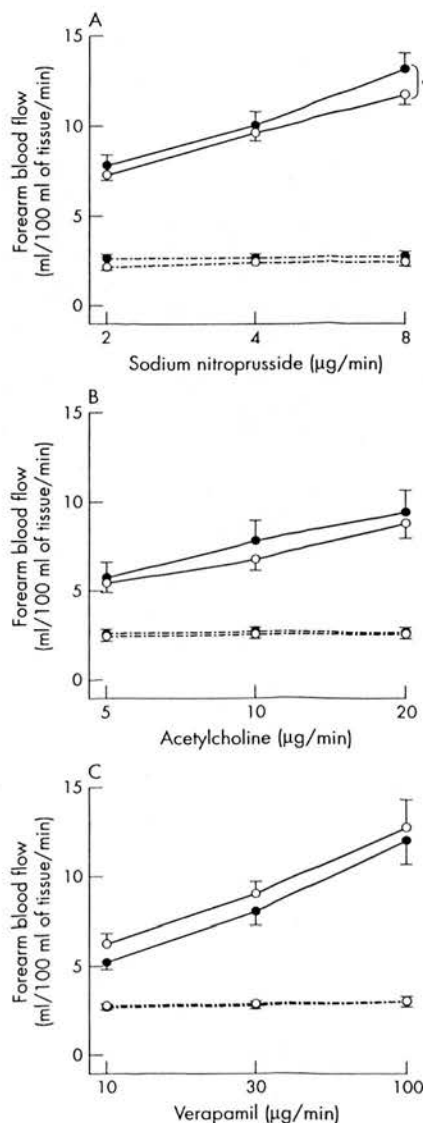


**Figure 2** Infused (solid line) and non-infused (dashed line) forearm blood flow in patients with CHD (●) and controls (○) during intrabrachial acetylcholine (panel A), sodium nitroprusside (panel B), and verapamil (panel C) with placebo infusion.  $p < 0.001$  analysis of variance, dose response in infused arm; \* $p = 0.005$  analysis of variance patients with CHD versus controls.

8 pmol/min, mean difference 0.3 ml/100 ml/min, 95% CI -0.1 to 0.7). However, sildenafil augmented the vasodilatation to sodium nitroprusside in both patients with CHD ( $p < 0.05$ , analysis of variance) (fig 3) and control subjects ( $p < 0.001$ , analysis of variance) (fig 4).

### Plasma fibrinolytic variables

Baseline plasma t-PA antigen concentrations were unchanged by sildenafil in either group (table 2, fig 5). Substance P caused a dose dependent increase in plasma t-PA concentrations in both patients and controls ( $p < 0.01$  for both, analysis of variance) (table 2). The substance P induced increase in plasma t-PA concentrations did not differ during the sildenafil or placebo infusion (at 8 pmol/min, mean difference 0.02 ng/ml, 95% CI -1.15 to 1.18) (table 2) and



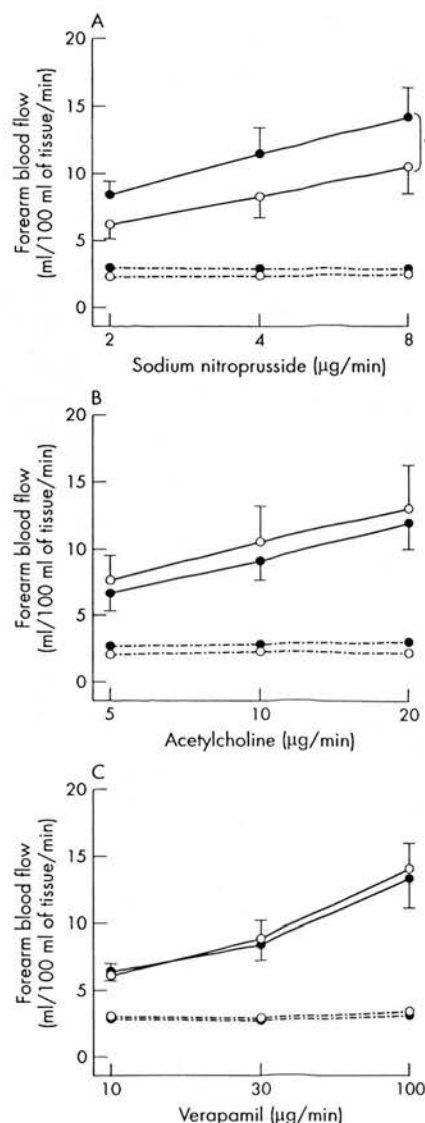
**Figure 3** Infused (solid line) and non-infused (dashed line) forearm blood flow in patients with CHD during intrabrachial sodium nitroprusside (panel A), acetylcholine (panel B), and verapamil (panel C) with sildenafil (●) and matched placebo (○) infusion.  $p < 0.001$  analysis of variance, dose response in infused arm; \* $p < 0.05$  analysis of variance, sildenafil versus matched placebo.

plasma PAI-1 concentrations did not change significantly throughout either study.

## DISCUSSION

We have shown that sildenafil, a selective PDE5 inhibitor, does not modify endothelium dependent vasodilatation or acute t-PA release in men with stable CHD. However, sildenafil did augment the vasodilator effect of the exogenous nitric oxide donor sodium nitroprusside. Thus, while our study confirms the well described interaction of sildenafil with nitric oxide donors,<sup>10,16</sup> we have found no evidence to support the contention that PDE5 inhibitors improve endothelium dependent vasomotor or fibrinolytic function in patients with CHD.

Compared with matched controls, patients with CHD exhibited impaired endothelium dependent responses to



**Figure 4** Infused (solid line) and non-infused (dashed line) forearm blood flow in healthy controls during intrabrachial sodium nitroprusside (panel A), acetylcholine (panel B), and verapamil (panel C) with sildenafil (●) and matched placebo (○) infusion.  $p \leq 0.01$  analysis of variance, dose response in infused arm; \* $p < 0.001$  analysis of variance, sildenafil versus matched placebo.

acetylcholine while having preserved vasodilator responses to the endothelium independent agonists sodium nitroprusside and verapamil. This prognostically significant impairment<sup>7,17</sup> was evident in patients who were already receiving standard antianginal, antiplatelet, and lipid lowering treatments.

Sildenafil had no effect on peak flow mediated dilatation of the brachial artery in patients with CHD<sup>11</sup> and reports on the vasomotor responses of the coronary vessels to sildenafil are conflicting. Herrmann *et al*<sup>18</sup> found no change in coronary artery diameter, blood flow, or coronary vascular resistance, whereas Halcox *et al*<sup>11</sup> reported enhanced coronary artery vasodilatation to acetylcholine. Unlike previous studies, we used a more robust double blind randomised placebo controlled crossover study design and have shown that PDE5 inhibition does not alter either endothelium dependent vasomotor or fibrinolytic function in patients with

**Table 2** Plasma tissue plasminogen activator (t-PA) and plasminogen activator inhibitor type 1 (PAI-1) concentrations at baseline and during sildenafil and matched placebo infusion in patients with coronary heart disease

Sildenafil/placebo Substance P dose (pmol/min)	0	Bolus	Continuous infusion (10 mg/h)		
			2	4	8
<b>Placebo</b>					
Plasma t-PA antigen (ng/ml)					
Infused arm	9.0 (0.7)	8.7 (0.6)	9.3 (0.6)	9.8 (0.7)	10.5 (0.8)*
Non-infused arm	9.0 (0.6)	9.1 (0.6)	9.1 (0.6)	8.9 (0.6)	9.1 (0.6)
Plasma PAI-1 antigen (ng/ml)					
Infused arm	46.8 (6.9)	43.1 (6.0)	0	0	34.4 (5.4)
Non-infused arm	45.2 (7.1)	45.5 (7.6)	0	0	36.4 (6.3)
<b>Sildenafil</b>					
Plasma t-PA antigen (ng/ml)					
Infused arm	9.0 (0.6)	8.7 (0.6)	9.1 (0.6)	9.4 (0.6)	10.5 (0.7)*
Non-infused arm	9.1 (0.6)	8.8 (0.6)	9.0 (0.6)	8.9 (0.6)	9.0 (0.6)
Plasma PAI-1 antigen (ng/ml)					
Infused arm	44.0 (5.6)	39.6 (4.6)	0	0	36.0 (3.8)
Non-infused arm	47.9 (5.6)	43.2 (4.9)	0	0	38.6 (4.2)

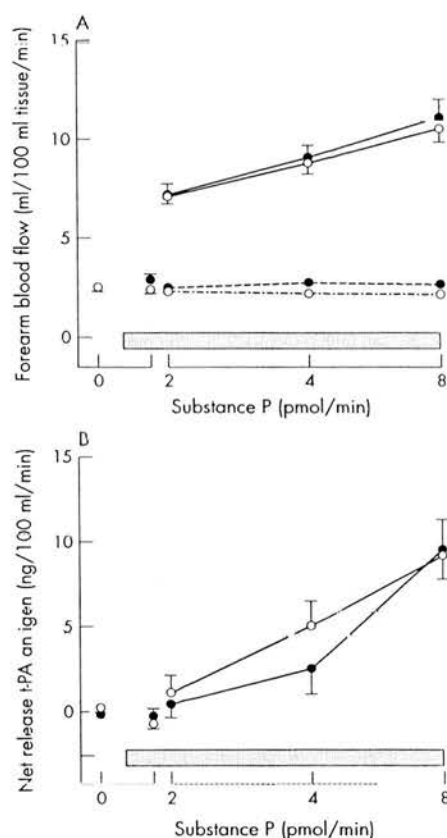
\* $p < 0.001$ , analysis of variance for t-PA response.Control subject data on file ( $p = 0.003$ , analysis of variance for t-PA response).

CHD or in age matched controls. Moreover, we used a bolus and continuous intravenous sildenafil infusion to minimise variations in plasma concentrations during the administration of each of the intra-arterial vasodilators. This

is an important study consideration given the short half life of sildenafil in humans.

We observed a decrease in mean arterial pressure in both patients and controls during administration of sildenafil that presumably reflected an augmentation of the vascular effects of basal vascular nitric oxide release and is mediated through an increase in cGMP. Our findings are consistent with the published haemodynamic data from both healthy volunteers<sup>19</sup> and patients with CHD<sup>18,20</sup> and confirm that we achieved a physiological effect with sildenafil infusion. The consistent vasodilatory response to the nitric oxide independent agonist verapamil makes it unlikely that administration of the PDE5 inhibitor impaired vascular smooth muscle function or obscured potentially beneficial effects on endothelial function. Moreover, both acetylcholine and substance P produced similar, consistent, and reproducible responses on both study days. This suggests that prolonging cGMP actions in patients with established atherosclerosis would not reverse endothelial dysfunction. As would be predicted from its mechanism of action, sildenafil augmented the responses to sodium nitroprusside, an exogenous nitric oxide donor, in both controls and patients with CHD.

There are several potential reasons for the differences observed in the effect of sildenafil on the acetylcholine and sodium nitroprusside responses. The modest decrease in acetylcholine induced vasodilatation<sup>21,22</sup> seen after nitric oxide synthase inhibition suggests that non-nitric oxide dependent pathways such as endothelium derived hyperpolarising factor (EDHF) may predominate particularly in the presence of endothelial dysfunction.<sup>23,24</sup> Furthermore, differences in the relative contribution of endothelium derived nitric oxide across vascular beds may explain some of the previously conflicting data on the vascular responses to sildenafil.<sup>11,25,26</sup> As well as endothelial dysfunction, atherosclerosis is associated with high concentrations of free radicals such as superoxide anion that rapidly react with nitric oxide to generate peroxynitrite, a powerful oxidant species that induces significant cellular damage and directly inhibits soluble guanylate cyclase.<sup>27</sup> Elegant studies in animals with specific knockouts of nitric oxide dependent pathways also suggest that tissue specific downregulation of nitric oxide/cGMP, including cGMP dependent protein kinase, may be an early feature of endothelial dysfunction in atherosclerotic conditions.<sup>28,29</sup> PDE5 inhibition would not be anticipated to influence changes in oxidative stress or directly affect cGMP independent nitric oxide molecular targets that contribute to endothelial dysfunction and



**Figure 5** Infused (solid line) and non-infused (dashed line) forearm blood flow (panel A) and estimated net release of tissue plasminogen activator (t-PA) antigen (panel B) at baseline, during sildenafil (●) and matched placebo (○) infusion (shaded box), and subsequently with intrabrachial substance P (2, 4, 8 pmol/min) in patients with CHD.  $p < 0.01$  analysis of variance for all, dose response for infused arm FBF and net t-PA release.



atherogenesis. Therefore, the contrasting effects of sildenafil on acetylcholine and sodium nitroprusside induced vasodilatation are likely to reflect a major dependence on non-nitric oxide mediated pathways, increased oxidative stress, and decreased nitric oxide bioavailability associated with CHD.

Although the precise mechanism underlying acute t-PA release remains uncertain, several reports have previously suggested involvement of nitric oxide and cyclic nucleotides regulated by phosphodiesterases. In animals, pentoxifylline and its analogues, non-selective phosphodiesterase inhibitors, increased acute t-PA release<sup>30</sup> and potentiated the effects of thrombolytic treatment.<sup>31</sup> We and others have reported acute endothelial t-PA release during intra-arterial substance P,<sup>32</sup> bradykinin,<sup>33</sup> and methacholine<sup>34</sup> infusions, as well as an inverse relation between acute t-PA release and atherosclerotic plaque burden within the coronary circulation.<sup>35</sup> In the present study, we have again shown a rise in both plasma t-PA antigen concentrations and net t-PA release with local intra-arterial substance P infusion. However, infusion of sildenafil did not change basal plasma t-PA concentrations or substance P induced t-PA release. Therefore, enhancement of cGMP apparently does not directly augment endothelial t-PA release in humans.

Although we found no such change in acute t-PA release with substance P, sildenafil may improve the response to other agonists such as bradykinin, which causes B2 receptor mediated prostacyclin and nitric oxide generation.<sup>35</sup> However, the dominant mechanism of t-PA release with bradykinin appears to be nitric oxide and prostacyclin independent with coupling of the B2 receptor to the calcium dependent G<sub>q</sub>/phospholipase C-beta pathway.<sup>36</sup> Some of the conflicting results on the effect of nitric oxide synthase inhibitors on t-PA release may suggest that there is physiological redundancy within the nitric oxide dependent pathways which contribute to the regulation of acute t-PA release in humans.

### Study limitations

In light of the haemodynamic changes seen in our study, intrabrachial infusion of sildenafil in sub-systemic locally active doses would be one approach to assess the direct vascular actions of PDE5 inhibition. However, sildenafil is metabolised by the liver to an active metabolite that accounts for nearly half of its phosphodiesterase inhibitory activity. Local intra-arterial infusion would not assess the action of this important metabolite. The effect of long term PDE5 inhibitor therapy remains unclear, and further studies with women and patients with diabetes mellitus would be of interest, as these groups may show differences within nitric oxide dependent pathways.<sup>17, 38</sup>

### Conclusion

Despite being highly effective in the management of erectile dysfunction, sildenafil does not modify endothelium dependent vasomotor or fibrinolytic function in patients with CHD. Phosphodiesterase inhibitors have already shown promise as novel treatments for conditions such as chronic heart failure<sup>39</sup> and pulmonary hypertension,<sup>40</sup> and these areas clearly warrant further research. However, on the basis of our results, we believe that PDE5 inhibitors are unlikely to reverse the generalised vascular dysfunction seen in patients with CHD.

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Competing interests: SDR has received financial support for attending scientific meetings from Pfizer Ltd; NAB was a member of a drug advisory committee evaluating sildenafil and has received and supervised research grants from Pfizer Ltd; DEN holds unrestricted educational grant awards and has undertaken paid consultancy for Pfizer Ltd.

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## IMAGES IN CARDIOLOGY

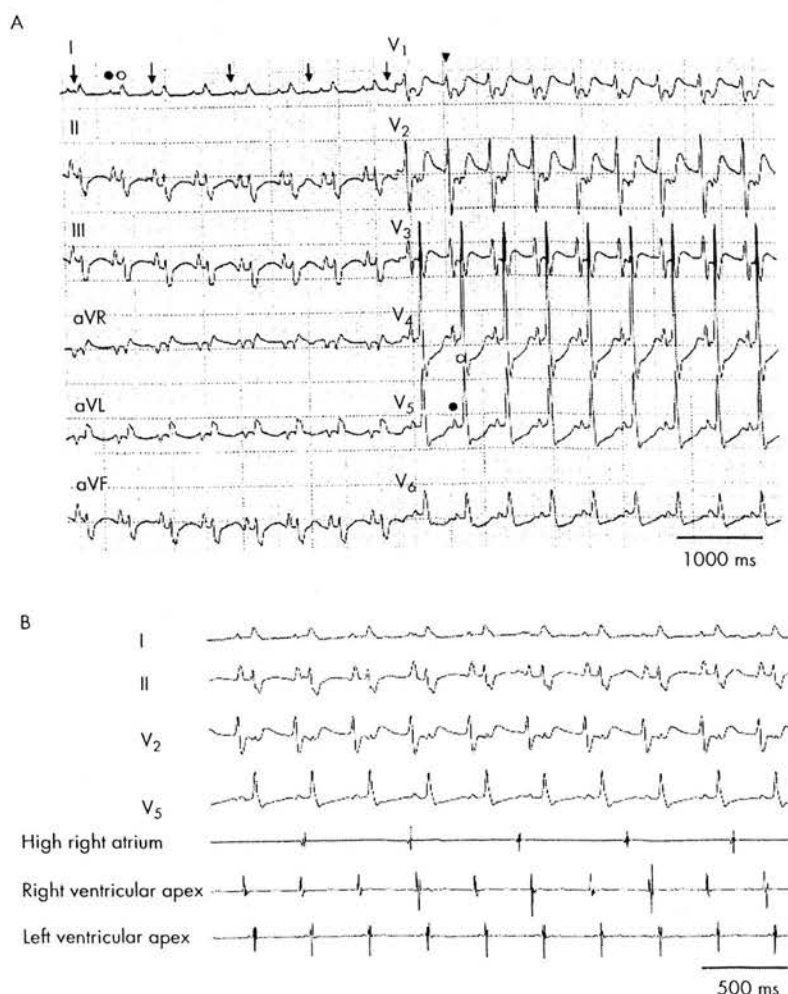
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### Pseudo-supraventricular tachycardia

A 79 year old man with dilated phase of hypertrophic cardiomyopathy and a history of anteroseptal myocardial infarction was admitted because of frequent episodes of tachyarrhythmia requiring cardioversion. A 12 lead ECG demonstrated small positive waves (closed circles) followed by larger positive waves (open circles) in limb leads and leads V4-6, which, at first glance, seemed to represent P waves followed by QRS complexes (panel A). A closer look indicated that the presumed P waves (closed circle) in leads V4-6 corresponded to R waves (arrowhead) in leads V1 and V2 as well as dissociation of true P waves (arrows) from QRS complexes. The diagnosis of ventricular tachycardia was confirmed by electrophysiologic study, which revealed that the first and second positive wave corresponded to the local ventricular potential recorded from the apex of right and left ventricle, respectively (panel B). Catheter ablation of the ventricular tachycardia was not possible because the tachycardia was not stable. The patient was treated with mexiletine and followed uneventfully.

This case re-emphasises the importance of recording a 12 lead ECG for the diagnosis of arrhythmia, even when the diagnosis from an ECG with fewer leads such as telemetry seems to be straightforward (sinus tachycardia or supraventricular tachycardia in this case).

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**PUBLICATION 22**

# Vascular and fibrinolytic effects of intra-arterial tumour necrosis factor- $\alpha$ in patients with coronary heart disease

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## A B S T R A C T

Elevated plasma t-PA (tissue plasminogen activator) and serum CRP (C-reactive protein) concentrations are associated with an adverse cardiovascular risk. In the present study, we investigated whether acute local inflammation causes vascular dysfunction and influences t-PA release in patients with stable coronary heart disease. Serum CRP, plasma t-PA and PAI-1 (plasminogen activator inhibitor type 1) concentrations were determined in 95 patients with stable coronary heart disease. A representative subpopulation of 12 male patients received an intra-brachial infusion of TNF- $\alpha$  (tumour necrosis factor- $\alpha$ ) and saline placebo using a randomized double-blind cross-over study design. Forearm blood flow and plasma fibrinolytic and inflammatory variables were measured. Serum CRP concentrations correlated with plasma t-PA concentrations ( $r = 0.37$ ,  $P < 0.001$ ) and t-PA/PAI-1 ratio ( $r = -0.21$ ,  $P < 0.05$ ). Intra-arterial TNF- $\alpha$  caused a rise in t-PA concentrations ( $P < 0.001$ ) without affecting blood flow or PAI-1 concentrations. TNF- $\alpha$  pretreatment impaired acetylcholine- and sodium nitroprusside-induced vasodilatation ( $P < 0.001$  for both) whilst doubling bradykinin-induced t-PA release ( $P = 0.006$ ). In patients with stable coronary heart disease, plasma fibrinolytic factors correlate with a systemic inflammatory marker and local vascular inflammation directly impairs vasomotor function whilst enhancing endothelial t-PA release. We suggest that the adverse prognosis associated with elevated plasma t-PA concentrations relates to the underlying causative association with vascular inflammation and injury.

## INTRODUCTION

In epidemiologic studies of patients with CHD (coronary heart disease) [1] and in prospective studies in healthy populations [2], higher plasma concentrations of the pro-fibrinolytic factor t-PA (tissue plasminogen activator)

positively and independently predict future cardiovascular events. It would be anticipated that high t-PA concentrations would protect against subsequent cardiovascular events rather than the reverse. This paradoxical association is, in part, explained by the concomitant elevation of PAI-1 (plasminogen activator inhibitor

**Key words:** coronary disease, endothelial function, fibrinolysis, inflammation, tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ), vasodilation.

**Abbreviations:** BP, blood pressure; CHD, coronary heart disease; CRP, C-reactive protein; F1 + 2, fragment 1 and 2; FBF, forearm blood flow; HR, heart rate; hs-CRP, highly sensitive CRP; IL-6, interleukin-6; IU, international units; NO, nitric oxide; eNOS, endothelial NO synthase; ns, not significant; PAI-1, plasminogen activator inhibitor type 1; SBP, systolic blood pressure; SNP, sodium nitroprusside; TNF- $\alpha$ , tumour necrosis factor- $\alpha$ ; t-PA, tissue plasminogen activator.

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type 1) which complexes with, and inactivates, t-PA. However, the precise stimulus for this increased t-PA release remains unclear.

Areas of endothelial denudation and thrombus deposition are a common finding on the surface of atherosclerotic plaques and are usually subclinical. Through t-PA release, endogenous fibrinolysis is usually able to prevent thrombus propagation, although organization of the residual thrombus may lead to plaque growth and expansion [3]. The adverse prognosis conferred by elevated plasma t-PA antigen concentrations may, therefore, reflect the extent of occult atheroma and subclinical plaque rupture stimulating t-PA release.

Markers of systemic inflammation, such as CRP (C-reactive protein) and TNF- $\alpha$  (tumour necrosis factor- $\alpha$ ), are elevated in patients with cardiovascular disease [4,5]. Indeed, serum CRP concentrations predict the development of cardiovascular disease independently of other risk factors. Previous studies have indicated a direct relationship between serum CRP and plasma t-PA concentrations [6,7]. This raises the question of whether vascular inflammation is causally related to the elevation in plasma t-PA concentrations or whether CRP and t-PA are independently increased by a common factor related to the atherosclerotic process itself, such as acute plaque rupture.

Abnormalities of endothelial function have been demonstrated in patients with atherosclerosis [8] and vascular inflammation [9,10]. In patients with CHD, restoration of endothelium-dependent vasomotor function occurs when there is normalization of CRP concentrations [11], whereas ongoing chronic inflammation is associated with an impaired fibrinolytic response to venous occlusion [12]. As the endothelium is the major source of plasma t-PA, abnormalities of endothelial function may therefore mediate the potential inflammation-induced elevations in plasma t-PA concentrations.

It therefore remains unclear whether elevated t-PA concentrations are implicated in the mechanisms contributing to, or arise as a consequence of, atherothrombotic events.

The aims of the present study were, in patients with stable CHD, to confirm the previous association between plasma CRP and t-PA concentrations and to determine the effect of acute local vascular inflammation provoked by direct intra-arterial infusion of TNF- $\alpha$  on vasomotor function and endothelial t-PA release.

## METHODS

### Patients

We recruited patients with CHD confirmed by angiography (defined as  $> 70\%$  luminal stenosis of at least one major epicardial coronary vessel) or a previous history of Q-wave myocardial infarction. All patients had stable

anginal symptoms and had not undergone coronary revascularization within the preceding 3 months. Exclusion criteria were significant cardiac failure, renal impairment, SBP [systolic BP (blood pressure)]  $< 100$  or  $> 190$  mmHg, diabetes mellitus, history or clinical features of recent infective illness and immunosuppressive or non-steroidal anti-inflammatory medication (excluding 75 mg/day aspirin). All studies were undertaken with the approval of the local Research Ethics Committee and in accordance with the Declaration of Helsinki. Written informed consent was obtained from each subject.

### Venous sampling and assays

Plasma t-PA, PAI-1 (Coalizer<sup>®</sup>; Chromogenix), prothrombin F1 + 2 (fragment 1 and 2; Enzygnost F1 + 2; Dade Behring), TNF- $\alpha$  (Quantikine; R&D Systems) and IL-6 (interleukin-6; Dako) concentrations were determined using ELISAs, and t-PA activity using a photometric method (Coatest t-PA; Chromogenix) [13–15]. Assays of hs-CRP (highly sensitive CRP) were undertaken using the method of particle-enhanced immunonephelometry (BN II nephelometer; Behring). Venous blood was collected into tubes containing acidified buffered citrate (for t-PA), trisodium citrate (for PAI-1 and prothrombin F1 + 2), potassium EDTA (for cytokines) and serum gel tubes (for CRP). Platelet-free plasma and serum were stored at  $-80^{\circ}\text{C}$  before assay. Haematocrit and white cell count were determined using an automated Coulter counter. Biochemical assays were undertaken on fasting venous samples by the hospital Clinical Laboratory facility.

### Drugs

TNF- $\alpha$  (Knoll Pharmaceuticals), bradykinin (Clinalfa), acetylcholine (Novartis) and SNP (sodium nitroprusside; David Bull Laboratories) were administered following dissolution in 0.9% saline.

### Study design

All subjects abstained from alcohol for 24 h and from food, tobacco and caffeine-containing drinks for at least 4 h before each study visit. A venous blood sample was taken from all patients for estimation of serum CRP and plasma t-PA and PAI-1 antigen concentrations. Twelve representative male patients were then recruited into a randomized double-blind placebo-controlled cross-over study comparing the effect of direct intra-brachial infusion of TNF- $\alpha$  and saline placebo.

### Forearm study protocol

All studies were carried out at 09:00 hours in a quiet temperature-controlled room maintained at  $22\text{--}25^{\circ}\text{C}$ . Patients rested recumbent and strain gauges and cuffs were applied. A 17-gauge venous cannula was inserted into the antecubital vein of each arm, and the brachial artery of the non-dominant arm was cannulated with a



27-SWG (standard wire gauge) needle (Cooper's Needle Works). FBF (forearm blood flow) was measured in both forearms by venous occlusion plethysmography using mercury in silastic strain gauges as described previously [16]. BP and HR (heart rate) were measured using a semi-automated non-invasive sphygmomanometer (Omron 705 IT).

Subjects ( $n = 12$ ) attended on two occasions at least two weeks apart and received an intra-arterial infusion of either TNF- $\alpha$  (80 ng/min) or saline placebo over 60 min, followed on each occasion by a further 60 min of saline infusion. Thereafter intra-arterial bradykinin (100, 300 and 1000 pmol/min), acetylcholine (5, 10 and 20  $\mu$ g/min) and SNP (2, 4 and 8  $\mu$ g/min) were administered at 1 ml/min with a 15 min saline washout period between each agent. The dose of TNF- $\alpha$  was chosen to achieve local cytokine concentrations comparable with healthy volunteer studies [13] and those seen in cardiovascular disease [17].

Venous blood samples for t-PA antigen and activity and PAI-1 antigen were obtained at baseline, after 60 min of TNF- $\alpha$ /placebo infusion, following the 60 min infusion of saline, before and during each dose of bradykinin and 15 min after the end of bradykinin infusion. Plasma cytokines, hs-CRP and prothrombin F1 + 2 were assessed prior to and following the TNF- $\alpha$ /placebo and saline infusions and at the end of each study.

## Statistical analysis

Estimated net release of t-PA was defined previously as the product of the infused forearm plasma flow and the concentration difference between the infused and non-infused forearms [16]. Because basal t-PA concentrations were altered by pretreatment with TNF- $\alpha$ , net release of t-PA during bradykinin infusion was calculated by subtracting the mean t-PA release before and 15 min after cessation of bradykinin infusion [13]. The area under the curve was calculated for the estimated net release of t-PA in response to bradykinin. Data were examined, where appropriate, by ANOVA with repeated measures, followed by post-hoc Student's *t* tests adjusted with a Bonferroni correction for multiple comparisons. Spearman's correlation was used to compare CRP and plasma levels of fibrinolytic factors. As serum CRP concentrations have a skewed distribution, they were logarithmically transformed.

All statistical calculations were undertaken using GraphPad Prism (GraphPad Software). Results are expressed as means  $\pm$  S.E.M., unless otherwise stated, and statistical significance was assigned at the 5% level.

## RESULTS

Patients had a typical cardiovascular risk factor profile in keeping with their diagnosis of CHD and most were

**Table 1** Baseline characteristics of the 95 subjects with stable CHD

Values are means  $\pm$  S.D. or *n* (%). ACE, angiotensin-converting enzyme; ARB, angiotensin II receptor blocker; LDL, low-density lipoprotein. \*Angiographic data were unavailable on one subject.

Characteristics	
Age (years)	59 $\pm$ 7
Body mass index (kg/m <sup>2</sup> )	29 $\pm$ 5
Male gender ( <i>n</i> )	79 (83%)
Previous myocardial infarction ( <i>n</i> )	39 (41%)
Extent of coronary artery disease ( <i>n</i> )*	
One vessel	42 (44%)
Two vessels	24 (25%)
Three vessels	28 (29%)
Previous coronary revascularization ( <i>n</i> )	64 (67%)
Co-morbidity ( <i>n</i> )	
Hypertension	46 (48%)
Previous hyperlipidaemia	89 (94%)
Family history of premature CHD	32 (34%)
Smoker/ex-smoker/non-smoker	30/27/38 (32%/28%/40%)
Medical therapy ( <i>n</i> )	
Aspirin	95 (100%)
Anti-anginal	82 (86%)
Statin	90 (95%)
ACE-inhibitor/ARB	30 (32%)
Serum urea (mmol/l)	5.6 $\pm$ 1.3
Serum creatinine ( $\mu$ mol/l)	93 $\pm$ 13
Plasma glucose (mmol/l)	5.7 $\pm$ 0.9
Total cholesterol (mmol/l)	4.5 $\pm$ 0.9
LDL-cholesterol (mmol/l)	2.5 $\pm$ 0.7
Triacylglycerols (mmol/l)	1.8 $\pm$ 0.9

prescribed secondary preventative medications (Tables 1 and 2). The majority of the subjects were male, two-thirds had previously undergone coronary revascularization and approx. one-third were habitual smokers.

## Plasma fibrinolytic factors and correlation with CRP

In the cohort of 95 patients with stable CHD, serum hs-CRP concentrations correlated with plasma t-PA antigen concentrations ( $r = 0.37$ ,  $P < 0.001$ ) and plasma PAI-1 antigen concentrations ( $r = 0.28$ ,  $P = 0.006$ ) and inversely with the ratio of t-PA/PAI-1 antigen ( $r = -0.21$ ,  $P < 0.05$ ). As anticipated, plasma t-PA antigen concentrations correlated with plasma PAI-1 antigen concentrations ( $r = 0.49$ ,  $P < 0.001$ ).

## Effect of acute inflammation on vascular and fibrinolytic function

Those subjects who received TNF- $\alpha$  and saline placebo had similar baseline characteristics to the main cohort and all were receiving statin and aspirin therapy. There were

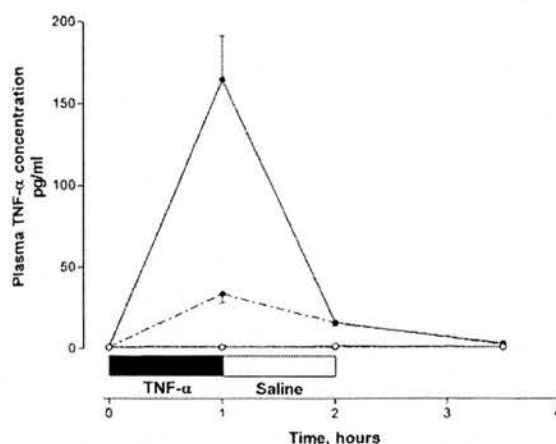
**Table 2** Baseline characteristics of 12 patients receiving TNF- $\alpha$  and saline placebo in a randomized double-blind cross-over study design

Values are means  $\pm$  S.E.M or *n* (%). ACE, angiotensin-converting enzyme; LDL, low-density lipoprotein. \*Angiographic data were unavailable on one subject.

Characteristics	
Age (years)	60 $\pm$ 2
Body mass index (kg/m <sup>2</sup> )	24 $\pm$ 1
Male gender ( <i>n</i> )	12 (100 %)
Previous myocardial infarction ( <i>n</i> )	5 (42 %)
Extent of coronary disease ( <i>n</i> )*	
One vessel	5 (42 %)
Two vessels	4 (33 %)
Three vessels	2 (17 %)
Previous coronary revascularization ( <i>n</i> )	9 (75 %)
Co-morbidity ( <i>n</i> )	
Hypertension	3 (25 %)
Family history of premature CHD	1 (8 %)
Previous hyperlipidaemia	12 (100 %)
Smoker/ex-smoker/non-smoker	2/7/3 (17 %/58 %/25 %)
Medical therapy ( <i>n</i> )	
Aspirin	12 (100 %)
Anti-anginal	11 (92 %)
Statin	12 (100 %)
ACE-inhibitor	1 (8 %)
Serum urea (mmol/l)	5.9 $\pm$ 0.3
Serum creatinine ( $\mu$ mol/l)	93 $\pm$ 2
Plasma glucose (mmol/l)	5.2 $\pm$ 0.1
Total cholesterol (mmol/l)	3.9 $\pm$ 0.2
LDL-cholesterol (mmol/l)	2.1 $\pm$ 0.2
Triacylglycerols (mmol/l)	1.3 $\pm$ 0.2
Placebo visit	
HR (beats/min)	56 $\pm$ 2
SBP (mmHg)	145 $\pm$ 6
Diastolic BP (mmHg)	83 $\pm$ 4
FBF (ml $\cdot$ 100 ml <sup>-1</sup> of tissue $\cdot$ min <sup>-1</sup> )	
Infused arm	2.1 $\pm$ 0.2
Non-infused arm	2.0 $\pm$ 0.2
TNF- $\alpha$ visit	
HR (beats/min)	56 $\pm$ 3
SBP (mmHg)	142 $\pm$ 4
Diastolic BP (mmHg)	81 $\pm$ 2
FBF (ml $\cdot$ 100 ml <sup>-1</sup> of tissue $\cdot$ min <sup>-1</sup> )	
Infused arm	2.0 $\pm$ 0.2
Non-infused arm	2.1 $\pm$ 0.2

no differences in resting arterial pressure, HR or FBF between the two study visits (Table 2).

There was no change in HR or BP during infusion of either TNF- $\alpha$  or placebo. Haematocrit, temperature, white cell count and hs-CRP were similar on both study visits [ $P$  = ns (not significant); results not shown]. The study protocol was well tolerated with no major adverse effects.



**Figure 1** Plasma TNF- $\alpha$  concentrations in the infused (solid line) and non-infused arm (dashed/dotted line) after 60 min of intra-arterial TNF- $\alpha$  (●) and saline placebo (○)

$P < 0.001$  for the TNF- $\alpha$  dose-response in the infused arm, as determined by ANOVA;  $P < 0.001$  when TNF- $\alpha$  compared with saline placebo in the infused arm, as determined by ANOVA.

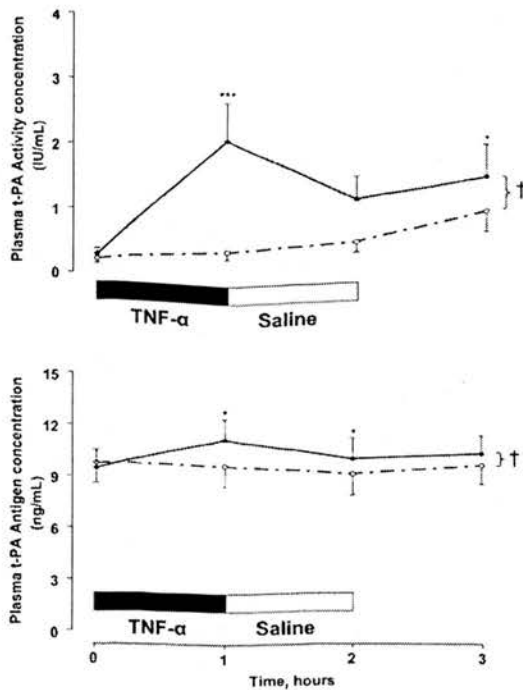
### Plasma cytokine concentrations

Intra-arterial infusion of TNF- $\alpha$  increased plasma TNF- $\alpha$  concentrations from  $1.4 \pm 0.2$  to  $164.5 \pm 26.8$  pg/ml in the infused arm and from  $1.3 \pm 0.1$  to  $33.3 \pm 5.6$  pg/ml in the non-infused arm ( $P < 0.001$ ; Figure 1). At 1 h after TNF- $\alpha$  infusion, plasma IL-6 concentrations increased from  $4.1 \pm 1.9$  to  $6.4 \pm 2.3$  pg/ml in the infused arm ( $P < 0.001$ , as determined by ANOVA), but were unchanged in the non-infused arm ( $4.0 \pm 1.9$  to  $3.7 \pm 1.3$  pg/ml). Placebo infusion had no effect on plasma IL-6 concentrations in the infused arm ( $3.9 \pm 1.7$  to  $4.3 \pm 1.3$  pg/ml;  $P$  = ns). Serum CRP concentrations were unchanged following the 60 min infusion of TNF- $\alpha$  and saline placebo.

### Vasomotor and fibrinolytic responses

There was no significant change in resting FBF in the 2 h after the start of either TNF- $\alpha$  or placebo infusion. After 60 min of TNF- $\alpha$ , plasma t-PA antigen and activity concentrations in the infused arm had increased from  $9.4 \pm 1.1$  to  $11.3 \pm 1.2$  ng/ml and  $0.3 \pm 0.1$  to  $2.1 \pm 0.6$  IU (international units)/ml respectively ( $P < 0.001$ ), and these remained elevated in the 2 h following discontinuation of the TNF- $\alpha$  infusion (Figure 2). There was no change in plasma PAI-1 antigen concentrations in the infused arm and no change in either plasma t-PA or PAI-1 concentrations in the non-infused arm during the study (results not shown). Prothrombin F1 + 2 concentrations in the infused arm were unaltered following either saline placebo ( $0.9 \pm 0.1$  to  $0.9 \pm 0.1$  ng/ml;  $P$  = ns) or TNF- $\alpha$  infusion ( $0.9 \pm 0.1$  to  $1.0 \pm 0.1$  ng/ml;  $P$  = ns).

There was a dose-dependent increase in FBF during bradykinin, acetylcholine and SNP infusion ( $P \leq 0.01$ ,



**Figure 2** Plasma t-PA activity (upper panel) and antigen (lower panel) concentrations in the infused (●) and non-infused (○) arms after 60 min infusion of intra-arterial TNF- $\alpha$

† $P < 0.001$  when infused compared with non-infused arms, as determined by ANOVA. \* $P < 0.05$  and \*\*\* $P < 0.001$ , as determined by post-hoc Student's  $t$  test for treatment effect.

as determined by ANOVA). Compared with the saline placebo, TNF- $\alpha$  pretreatment impaired acetylcholine- and SNP-induced vasodilatation ( $P < 0.001$  for both), but did not alter the response to bradykinin (Figure 3).

Plasma t-PA concentrations increased in a dose-dependent manner during bradykinin infusion on both study

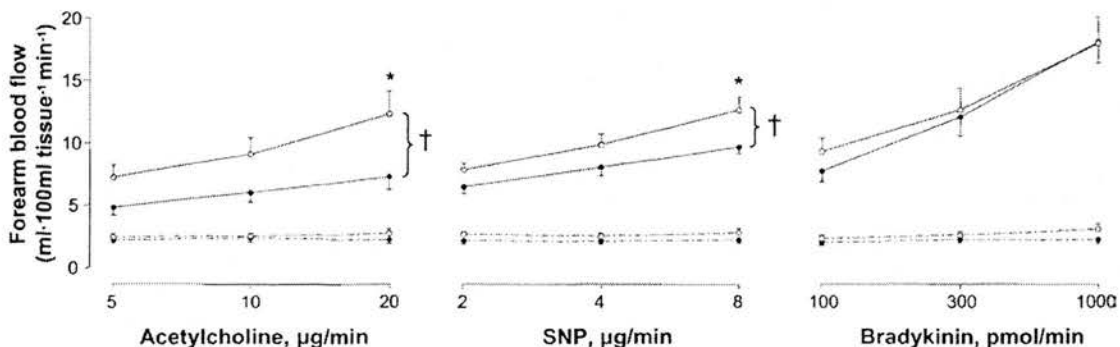
visits ( $P < 0.001$ ). Pretreatment with TNF- $\alpha$  augmented the bradykinin-induced rise in plasma t-PA antigen and activity concentrations ( $P < 0.001$  for both; Figure 4) and significantly increased estimated net release of t-PA antigen ( $63.7 \pm 14.8$  compared with  $120.6 \pm 26.1$  ng · 100 ml $^{-1}$  of tissue · min $^{-1}$  at peak dose;  $P < 0.05$ ) and activity ( $54.8 \pm 14.8$  compared with  $98.8 \pm 21.0$  IU · 100 ml $^{-1}$  of tissue · min $^{-1}$  at peak dose;  $P < 0.05$ ). Over the 30 min period of bradykinin infusion, TNF- $\alpha$  increased the area under the curve for net t-PA antigen and activity release by 120 % and 188 % respectively ( $P = 0.006$ ).

Subgroup analysis showed a significant impairment of endothelium-dependent vasodilatation as well as bradykinin-induced t-PA response in cigarette smokers. Qualitatively the effect of intra-arterial TNF- $\alpha$  on the blood flow and fibrinolytic responses was similar in both smokers and non-smokers.

## DISCUSSION

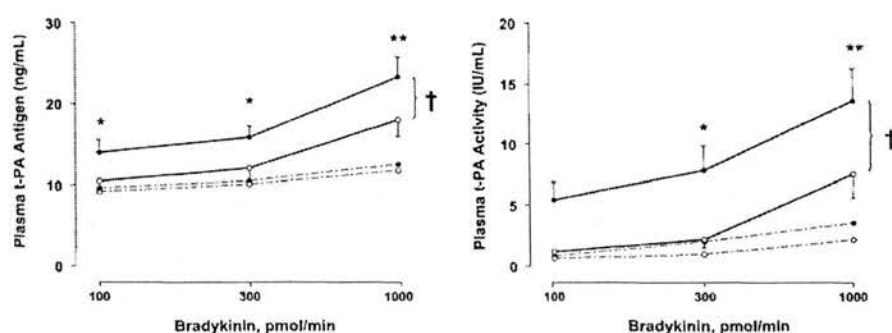
In the present study, we have confirmed the direct association between plasma t-PA and serum CRP concentrations in patients with stable CHD. For the first time, we have extended this observation using an acute local vascular inflammatory model and demonstrated that direct intra-arterial infusion of TNF- $\alpha$  causes a slow onset and sustained increase in basal t-PA release. This arterial inflammation was also associated with increased stimulated t-PA release in the presence of impaired vasomotor function. Our findings are consistent with the suggestion that t-PA is released during vascular inflammation and endothelial injury and this may, in part, explain the adverse prognosis associated with increased plasma t-PA concentrations.

The link between markers of inflammation and plasma t-PA concentrations suggests that vascular inflammation and injury may be responsible for endothelial t-PA



**Figure 3** Infused (solid line) and non-infused (dashed/dotted line) FBF during incremental doses of acetylcholine (left-hand panel), SNP (middle panel) and bradykinin (right-hand panel) following pretreatment with TNF- $\alpha$  (●) or saline placebo (○)

$P \leq 0.01$  for all infused arm responses, as determined by ANOVA. † $P < 0.001$  when TNF- $\alpha$  compared with saline placebo treatments, as determined by ANOVA. \* $P < 0.05$ , as determined by post-hoc Student's  $t$  test for treatment effect.



**Figure 4** Plasma concentrations of t-PA antigen (left-hand panel) and t-PA activity (right-hand panel) during bradykinin infusion following pretreatment with TNF- $\alpha$  (●) or saline placebo (○)

† $P < 0.001$  when TNF- $\alpha$  compared with saline placebo treatments, as determined by ANOVA. \* $P < 0.05$  and \*\* $P < 0.01$ , as determined by post-hoc Student's  $t$  test for treatment effect.

release. However, this association may arise from common aetiological factors and does not establish a causal relationship. A recent meta-analysis has suggested that increases in plasma t-PA concentrations largely reflect the presence of concomitant cardiovascular risk factors [7]. We therefore sought to induce vascular inflammation in a representative sample of our study population. Inducing systemic inflammation will have many biological actions and could be confounded by indirect or extra-vascular effects. This may explain some of the differences between our present results and those of previous work in healthy volunteers using *Salmonella typhi* vaccination [9,10]. We chose to employ an acute local inflammatory model [13] to assess the direct effects of intra-arterial TNF- $\alpha$  administration on t-PA release. We were able to induce local vascular inflammation with a rise in local plasma IL-6 concentrations to levels comparable with those seen in patients with unstable angina [17], without evidence of a systemic inflammatory response or change in plasma t-PA or PAI-1 concentrations in the non-infused arm. This local vascular inflammation caused a slow onset and sustained increase in plasma t-PA concentrations that continued for at least 2 h after cessation of the TNF- $\alpha$  infusion. This establishes that vascular inflammation directly causes endothelial t-PA release in humans.

The mechanism of TNF- $\alpha$ -induced t-PA release has not been established. Inflammatory cytokines, such as TNF- $\alpha$ , may cause t-PA release via induction of local thrombus formation, activation of specific cellular receptors or through generation of secondary mediators within the local vasculature. The former seems unlikely given that we observed no increase in prothrombin F1 + 2, a sensitive marker of *in vivo* thrombin generation [15].

Although it is likely that plasma t-PA concentrations are increased by inducing endothelial injury, smooth muscle cells, macrophages and monocytes also express t-PA mRNA following stimulation by inflammatory cytokines within atherosclerotic plaques [18] and could theoretically contribute to this fibrinolytic response.

### Effects of TNF- $\alpha$ on vasomotor function

Impaired vasodilator responses to acetylcholine [19,20] and SNP [20] in patients with cardiovascular risk factors predict an increased risk of adverse cardiovascular events. Previous studies in healthy volunteers have shown acute systemic inflammation is associated with a transient impairment in vasomotor function [9]. In the present study, we have now shown that, in patients with CHD, acute local vascular inflammation decreases the vasomotor response to both acetylcholine and SNP. Acetylcholine is known to stimulate NO (nitric oxide) production via activation of eNOS (endothelial NO synthase) and, together with the impaired SNP response, these findings suggest that local arterial inflammation can decrease NO bioavailability. Interestingly, impaired vasodilator responses to both acetylcholine and SNP have been correlated with plasma TNF- $\alpha$  concentrations in patients with rheumatoid arthritis, a chronic inflammatory condition which is itself associated with an excess cardiovascular risk [21]. Furthermore, intra-arterial infusion of the free radical scavenger vitamin C restores forearm blood flow responses in patients with CHD and elevated serum levels of CRP [22].

Our results suggest that acute inflammation is associated with impaired NO-dependent smooth muscle relaxation in response to direct NO donors such as SNP or endogenously derived NO following stimulation of eNOS. Several lines of evidence support the contention that cytokines, such as TNF- $\alpha$ , may impair NO-dependent signalling. TNF- $\alpha$  decreases eNOS expression [23] as well as increasing reactive oxygen species, such as superoxide anion, that rapidly inactivate NO and are directly cytotoxic to vascular tissues. TNF- $\alpha$  also increases plasma ADMA (asymmetric dimethylarginine), an endogenous inhibitor of eNOS that inhibits endothelium-dependent vasodilatation [24]. Finally, inflammatory states may increase iNOS (inducible NO synthase) expression which is associated with receptor uncoupling and endothelial dysfunction [25].

Bradykinin-induced vasodilatation was unaltered by pretreatment with TNF- $\alpha$ . Previous work has suggested that NO contributes only a small proportion (approx. 15%) to bradykinin-induced vasodilatation [26,27] and does not contribute to the mechanism of bradykinin-induced t-PA release [28]. Moreover, in patients with vascular dysfunction, there may be an increased contribution of EDHF (endothelium-derived hyperpolarizing factor) to smooth muscle vasorelaxation [29], particularly if NO is consumed by free radicals generated by locally active inflammatory cells. These observations are consistent with our present findings of impaired acetylcholine- and SNP-induced vasodilatation, but preserved bradykinin responses.

### Effects of TNF- $\alpha$ on acute endogenous fibrinolysis

Despite the presence of higher baseline plasma t-PA concentrations in patients with atherosclerosis [7], we have shown in the present study that direct intra-arterial TNF- $\alpha$  infusion increases bradykinin-induced t-PA release in patients with stable CHD. Thus, although vasorelaxation was impaired, acute inflammation initiates a sustained increase in both basal and stimulated t-PA release. The mechanism of this effect is unknown, but may involve direct endothelial injury, up-regulation of t-PA synthesis or alterations in bradykinin receptor expression.

Under some circumstances, increases in t-PA may protect against the propagation of intravascular thrombosis and thereby avoid the development of an acute coronary syndrome. However, elevations in plasma t-PA concentrations may reflect more widespread endothelial dysfunction and a dominant pro-inflammatory vascular response that may overwhelm any locally protective pro-fibrinolytic effect. Indeed, the pro-fibrinolytic actions of vascular inflammation may potentiate degradation of extracellular matrix and aggravate plaque instability [30]. The clinical outcome of acute vascular inflammation may, therefore, depend upon the relative balance between the protective antithrombotic actions and potential plaque destabilization associated with increased vascular t-PA release.

In the present study, all patients who received TNF- $\alpha$  and placebo infusion were already receiving secondary preventative therapy, including aspirin and lipid-lowering medications. Although these may have influenced the vascular response to TNF- $\alpha$ , it was considered unethical to withhold these and, in clinical practice, a large proportion of patients presenting with acute coronary syndromes and raised inflammatory markers are already established on such therapies. As our study design was focused on the question of the link between vascular inflammation and t-PA release in patients with CHD, we did not include a control population of healthy subjects. However, we have shown previously [13] that intra-

arterial TNF- $\alpha$  enhances endothelium-dependent t-PA release by a similar degree in younger healthy volunteers. Although we have again demonstrated that smokers have impaired endothelial responses, including t-PA release [14], intra-arterial TNF- $\alpha$  increased plasma t-PA concentrations to a similar degree in both smokers and non-smokers with established CHD.

We have found that, although TNF- $\alpha$  adversely affects NO-dependent vasodilatation, it enhances other protective mechanisms, such as the endogenous fibrinolytic capacity. This reflects the complex and pleiotropic nature of TNF- $\alpha$  which functions as part of the normal host surveillance mechanisms and response to tissue injury. Although we only determined the effect of acute vascular inflammation in 12 patients, our results may explain some of the contradictory findings of previous clinical studies. For example, in patients with heart failure, TNF- $\alpha$  antagonism causes marked improvements in endothelium-dependent vasodilatation [31], but has failed to demonstrate clinical benefit in randomized controlled trials [32]. Thus the benefits of restoring endothelium-dependent vasomotor function by TNF- $\alpha$  antagonism may be counterbalanced by inhibiting other potentially beneficial acute effects, such as enhancing endogenous t-PA release.

### Conclusions

In the present study, we have shown that, in patients with stable CHD, plasma fibrinolytic factors are correlated with CRP, a sensitive and prognostically relevant marker of vascular inflammation. We have also demonstrated that acute vascular inflammation directly impairs vasomotor function whilst enhancing endothelial t-PA release. We suggest that the adverse prognosis associated with elevated plasma t-PA concentrations reflects a causative association with vascular inflammation and injury, rather than representing a marker of occult plaque rupture.

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**PUBLICATION 23**



REGULAR ARTICLE

# Plasma TAFI and soluble CD40 ligand do not predict reperfusion following thrombolysis for acute myocardial infarction

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## KEYWORDS

Fibrinolysis;  
Myocardial infarction;  
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## Abstract

**Introduction:** Thrombolytic therapy fails to achieve reperfusion in almost a third of patients with acute myocardial infarction. Thrombin activatable fibrinolysis inhibitor (TAFI) and soluble CD40 ligand (sCD40L) are novel endogenous fibrinolytic and atherothrombotic factors that determine clot stability. We investigated whether admission plasma thrombin activatable fibrinolysis inhibitor (TAFI) and soluble CD40 ligand (sCD40L) concentrations predicted reperfusion following thrombolytic therapy in patients with acute myocardial infarction.

**Materials and methods:** Prior to administration of thrombolytic therapy, venous blood was collected from 110 patients presenting with acute ST segment elevation myocardial infarction and plasma assayed for tissue plasminogen activator (t-PA) antigen and activity, plasminogen activator inhibitor type-1 antigen (PAI-1), TAFI antigen and activity, C-reactive protein (CRP) and sCD40L concentrations. Reperfusion was determined using continuous ST segment monitoring.

**Results:** Reperfusion occurred in 77 (70%) patients with a mean treatment to reperfusion time of  $83 \pm 46$  min. Peak creatine kinase was significantly lower in patients who reperfused ( $1578 \pm 1199$  versus  $2200 \pm 1744$  U/L;  $P < 0.05$ ) and correlated with time to reperfusion ( $r = 0.44$  [95% CI: 0.23 – 0.61],  $P = 0.0001$ ).

**Abbreviations:** CRP, C-reactive protein; ELISA, Enzyme-linked immunosorbent assay; PAI-1, Plasminogen activator inhibitor type-1; sCD40L, Soluble CD40 ligand; t-PA, Tissue-type plasminogen activator; TAFI, Thrombin activatable fibrinolysis inhibitor.

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There was a modest correlation between plasma TAFI antigen and activity ( $r=0.3$  [95% CI: 0.04 – 0.53];  $P<0.05$ ). There were no significant associations between coronary reperfusion and plasma concentrations of t-PA, PAI-1, TAFI, CRP or sCD40L. *Conclusions:* Systemic plasma TAFI, sCD40L and CRP concentrations do not predict reperfusion in patients receiving thrombolytic therapy for acute ST elevation myocardial infarction.

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## Introduction

Thrombolytic therapy is an established treatment for acute ST elevation myocardial infarction [1]. In almost a third of patients thrombolysis fails to achieve timely reperfusion of the infarct-related artery and this is associated with a greater mortality and morbidity [2]. Arterial thrombosis is a complex, dynamic process that is dependent on a number of variables including endogenous fibrinolytic activity, vascular inflammation and platelet activation. These latter factors may play an important role in determining successful reperfusion following thrombolytic therapy.

Endogenous fibrinolytic activity is largely dependent on the balance between tissue plasminogen activator (t-PA) and its inhibitor, plasminogen activator inhibitor type-1 (PAI-1) [3]. In both healthy individuals [4] and patients with cardiovascular disease [5], reduced plasma fibrinolytic activity is associated with an increased risk of myocardial infarction. Evidence that the endogenous fibrinolytic system may be important in the acute phase of myocardial infarction is exemplified by the observation that in a third of patients, the infarct-related artery spontaneously reperfused within 12 h [6]. Moreover, baseline plasma PAI-1 concentrations appear to predict reperfusion following thrombolysis [7,8].

Thrombin activatable fibrinolysis inhibitor (TAFI) is a recently described pro-carboxypeptidase that, once activated, removes the C-terminal lysine residues from partially degraded fibrin, thereby preventing the binding of plasminogen and inhibiting fibrinolysis [9]. Thrombin activatable fibrinolysis inhibitor circulates in plasma as an inactive precursor but in the presence of the thrombin–thrombomodulin complex undergoes proteolytic conversion to its active form, TAFIa [9]. In man, plasma TAFI antigen concentration is largely under genetic control [10] and elevated concentrations have been reported in patients with stable ischemic heart disease, although this finding has not been universal [11,12]. In animal models, inhibition of TAFI potentiates t-PA mediated fibrinolysis [13], and in healthy man, plasma TAFI correlates with clot lysis time in vitro [14].

Inflammation, fibrinolysis and thrombosis are intimately interlinked [15]. C-reactive protein (CRP) is an established marker of inflammation that has pro-thrombotic actions. In patients with acute myocardial infarction, increased plasma CRP concentrations are associated with a poorer outcome [16] and appear to predict successful reperfusion following thrombolytic therapy [17]. Novel inflammatory mediators, such as CD40/CD40 ligand dyad, have recently been described. CD40 ligand, a member of the tumour necrosis factor family, is expressed along with its receptor, CD40, on a wide range of atheroma-associated cells [18]. In addition to the cell-associated form, CD40 ligand is also present in plasma as the biologically active fragment, soluble CD40 ligand (sCD40L) [18]. The CD40/CD40 ligand dyad mediates a wide range of pro-inflammatory and pro-thrombotic processes [18] including expression of cytokines, chemokines, cell adhesion molecules and tissue factor [19]. In addition, sCD40L acts as a platelet agonist [20] and is necessary for the stability of arterial thrombi [21]. As with CRP, plasma sCD40L concentrations are associated with an increased risk of recurrent events in patients with acute coronary syndromes [22].

The aim of this study was to establish whether the novel fibrinolytic and vascular inflammatory factors, TAFI and sCD40L, as well as plasma t-PA, PAI-1 and CRP concentrations are associated with successful reperfusion following thrombolytic therapy in patients with acute ST elevation myocardial infarction.

## Materials and methods

### Patients

One hundred and ten consecutive patients presenting with acute ST elevation myocardial infarction fulfilling the European Society of Cardiology clinical and electrocardiographic criteria for thrombolytic therapy [1] in whom informed consent was obtained were recruited. Patients with severe intercurrent illness, overt malignancy, active inflammatory disease or left bundle branch block on the electrocardiogram at presentation

were excluded. The study was performed with the approval of the local research ethics committee in accordance with the Declaration of Helsinki.

### Protocol

Immediately prior to administration of thrombolytic therapy (streptokinase or recombinant tissue-type plasminogen activator), 10 mL venous blood was collected from an antecubital vein using a 21-gauge needle and continuous ST segment monitoring was initiated. Where possible, streptokinase (1.5 million units) was the thrombolytic agent of choice. Recombinant t-PA (100 mg) was administered in place of streptokinase when patients had previously received streptokinase, had a systolic blood pressure <90 mm Hg or had evidence of extensive anterior infarction on the admission electrocardiogram. All patients received three hundred milligrams of aspirin prior to thrombolysis. Reperfusion of the infarct-related artery was determined non-invasively as a fall in ST segment elevation  $\geq 50\%$  or the onset of idioventricular rhythm [23,24].

### Blood samples and assays

Blood was collected into acidified buffered citrate (Biopool Stabilyte, Umeå; for t-PA assays) and citrate (Monovette, Sarstedt; for PAI-1, CRP, TAFI and sCD40L). Samples were kept on ice before being centrifuged at 2000  $\times g$  for 30 min at 4 °C. Platelet-free supernatant was decanted and stored at -80 °C before assay. Plasma t-PA antigen and activity, and PAI-1 antigen concentrations were determined using enzyme-linked immunosorbent assay (ELISA) and a photometric method as previously described [25]. The intra-assay coefficients of variation were 5.5% and 2.4% for t-PA antigen and activity, respectively and 7.0% for PAI-1 antigen. The inter-assay coefficients of variation were 4.0%, 4.0% and 7.3%, respectively. The sensitivities of the assays were 0.5 ng/mL, 0.1 IU/mL and 2.5 ng/mL, respectively.

Plasma CRP concentrations were measured using ultra-sensitive particle enhanced immunonephelometry (Dade Behring UK Ltd). Plasma TAFI antigen (Hyphen Biomed) and sCD40L (Bender MedSystems) concentrations were determined using commercially available ELISA kits. The total amount of activatable TAFI present in plasma was measured using a commercially available chromogenic assay (Actichrome). Plasma TAFI antigen concentrations are expressed as a percentage of normal pooled plasma. For technical reasons, plasma TAFI antigen and activity and sCD40L concentrations were only

available for the latter 56 patients enrolled in the study. The intra-assay coefficients of variation were 2% for CRP, 5% for TAFI antigen, 6% for TAFI activity and 7% for sCD40L. The inter-assay coefficients of variation were 4%, 5%, 9% and 14%, respectively. The sensitivities of the assays were 1 mg/L, 1.4%, 0.2  $\mu\text{g/mL}$  and 12 pg/mL, respectively.

Based on previous data [25,26,27], the present study had 80% power to detect a difference of 0.6 ng/mL, 0.3 IU/mL, 8 ng/mL, 0.1 pg/mL and 0.7  $\mu\text{g/mL}$  with a significance level of  $P < 0.05$  for t-PA antigen and activity, PAI-1 antigen, sCD40L and TAFI activity, respectively.

### Statistical analysis

Data are reported as mean  $\pm$  standard deviation for normally distributed data and median [interquartile range] for non-Gaussian data. Statistical analysis was performed using the chi-squared test, unpaired *t*-test for Gaussian data, and the Mann-Whitney *U* test for non-Gaussian data. The relationship between variables was assessed by linear regression analysis and is expressed as coefficient, significance value and the 95% confidence intervals for this relationship.

### Results

Patients were predominantly male with a mean age of 66 years (Table 1). Twenty-six (24%) patients were receiving treatment for hypertension. Ten patients (9%) had a previous diagnosis of diabetes mellitus and 46 patients (42%) were current smokers. Anterolateral ST segment elevation was present in 40 patients (36%) and inferior elevation in 70 (64%). Intravenous streptokinase was administered in 70 patients (64%) and recombinant t-PA in 40 (36%). There was no difference in the percentage of patients that reperfused following treatment with streptokinase compared to recombinant t-PA (48 (69%) versus 29 (73%) patients,  $P = 0.7$ ). There were no differences in the time of admission between the reperfusion and no reperfusion groups (Table 1).

Reperfusion of the infarct-related artery occurred in 77 (70%) patients (Table 1). The mean time from initiation of thrombolysis to reperfusion was  $83 \pm 46$  min. There was no difference in the time from onset of symptoms to treatment between the reperfusion and no reperfusion groups ( $230 \pm 180$  versus  $231 \pm 201$  min, respectively,  $P = 1.0$ ). Peak plasma creatine kinase concentrations were lower with reperfusion compared to no reperfusion ( $1578 \pm 1199$  versus  $2200 \pm 1744$  U/L, respectively,  $P < 0.05$ ,  $n = 110$ ;  $1690 \pm 1317$  versus



**Table 1** Patient demographics for all 110 patients and for the 56 patients in whom plasma soluble CD40 ligand and thrombin activatable fibrinolysis inhibitor concentrations were measured

	All (n=110)	Reperfusion (n=77)	No reperfusion (n=33)	P*	All (n=56)	Reperfusion (n=37)	No reperfusion (n=19)	P*
Age, years (range)	66 (37–86)	65 (37–82)	69 (48–86)	0.1	68 (37–84)	66 (37–79)	71 (50–84)	0.2
Male, n (%)	71 (65)	53 (69)	18 (54)	0.2	34 (61)	25 (68)	9 (47)	0.14
Time to treatment (min)	230 ± 186	230 ± 180	231 ± 201	1.0	243 ± 207	232 ± 204	265 ± 215	0.6
Risk factors, n (%)								
Hypertension	26 (24)	19 (25)	7 (21)	0.2	12 (21)	9 (24)	3 (16)	0.5
Diabetes mellitus	10 (9)	6 (8)	4 (12)	0.5	5 (9)	2 (5)	3 (16)	0.2
Current smoker	46 (42)	33 (43)	13 (39)	0.2	18 (32)	11 (30)	7 (37)	0.6
Prior aspirin use	30 (27)	21 (27)	9 (27)	1.0	17 (30)	11 (30)	6 (32)	0.9
Time of treatment, n (%)								
00:00–08:00	16 (15)	13 (17)	3 (9)	0.3	4 (7)	3 (8)	1 (5)	0.7
08:00–16:00	66 (60)	45 (58)	21 (64)	0.6	41 (73)	25 (68)	16 (84)	0.2
16:00–24:00	18 (25)	19 (25)	9 (27)	0.7	11 (20)	9 (24)	2 (11)	0.2

Data are expressed as mean ± SD or as indicated. \*Reperfusion versus no reperfusion (unpaired *t* test or chi squared).

2715 ± 1983, respectively,  $P < 0.05$ ,  $n = 56$ ) and correlated with the time to reperfusion ( $r = 0.44$  [95% CI: 0.23 to 0.61],  $P = 0.0001$ ,  $n = 110$ , Fig. 1;  $r = 0.53$  [95% CI: 0.25 to 0.73],  $P < 0.05$ ,  $n = 56$ ).

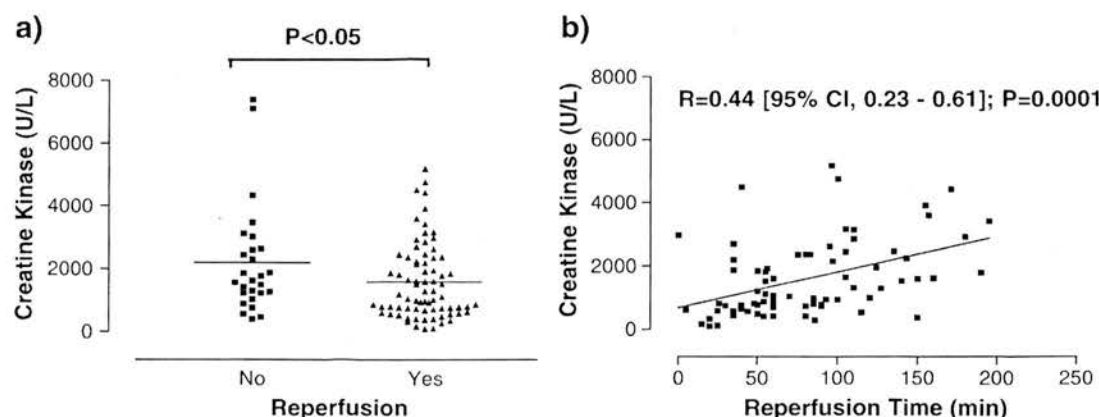
### Fibrinolytic factors

There were no differences in plasma concentrations of t-PA antigen and activity, TAFI antigen and activity, and PAI-1 antigen between the reperfusion and no reperfusion groups (Table 2). There were no associations between time to reperfusion and plasma concentrations of t-PA, PAI-1 and TAFI (Fig. 2). For patients enrolled within six hours of the onset of symptoms, there were no differences in plasma t-PA, PAI-1 and TAFI concentrations between the reperfusion and no reperfusion groups (data not shown). There was a modest

correlation between TAFI antigen and activity ( $r = 0.3$ , [0.04–0.53];  $P < 0.05$ ; Fig. 2).

### C-reactive protein and soluble CD40 ligand

There were no differences in plasma CRP or sCD40L concentrations between the reperfusion and no reperfusion groups (Table 3). Plasma CRP concentrations were significantly lower in patients presenting within six hours of symptom onset compared to those with symptoms of greater than six hours duration (1.6 [0.8–4.0] versus 4.5 [1.2–19.5] mg/L, respectively;  $P < 0.01$ ). There were no associations between time to reperfusion and either plasma CRP (data not shown) or sCD40L (Fig. 2) concentrations. There were no differences in plasma concentrations of CRP (Table 3) or



**Figure 1** (a) Peak serum creatine kinase concentrations in patients who do (triangles) and do not (squares) reperfuse following thrombolytic therapy, and (b) correlation between time to reperfusion and peak serum creatine kinase concentrations ( $R = 0.44$  [95% CI: 0.23 – 0.61];  $P = 0.0001$ ).

**Table 2** Plasma concentrations of endogenous fibrinolytic factors in patients presenting with acute ST elevation myocardial infarction

	All	Reperfusion	No reperfusion	P*	Difference* [95% CI]
t-PA antigen (ng/mL)	14.3 ± 6.8	14.3 ± 0.8	14.0 ± 5.9	0.8	−0.3 [−2.7 to 3.3]
t-PA activity (IU/mL)	1.6 ± 3.0	1.7 ± 3.2	1.6 ± 2.3	1.0	0.0 [−1.3 to 1.2]
PAI-1 antigen (ng/mL)	72 ± 32	73 ± 31	69 ± 34	0.6	−3.2 [−10.1 to 16.4]
t-PA:PAI-1 ratio	0.3 ± 0.3	0.2 ± 0.3	0.3 ± 0.3	0.2	0.0 [−0.1 to 0.1]
TAFI antigen (%)†	117 ± 20	118 ± 29	116 ± 24	0.8	−1.7 [−13.8 to 17.1]
TAFI activity (µg/mL)†	14.5 ± 3.3	14.5 ± 3.4	14.4 ± 3.3	0.9	−0.1 [−1.8 to 2.1]

Data are expressed as mean ± SD. The difference between means [95% Confidence Interval (CI)] is also given. t-PA, tissue plasminogen activator; PAI-1, plasminogen activator inhibitor type 1; TAFI, thrombin activatable fibrinolysis inhibitor. \*Reperfusion versus no reperfusion (unpaired *t* test). † (*n* = 56).

sCD40L (data not shown) between the reperfusion and no reperfusion groups for patients enrolled within six hours of the onset of symptoms.

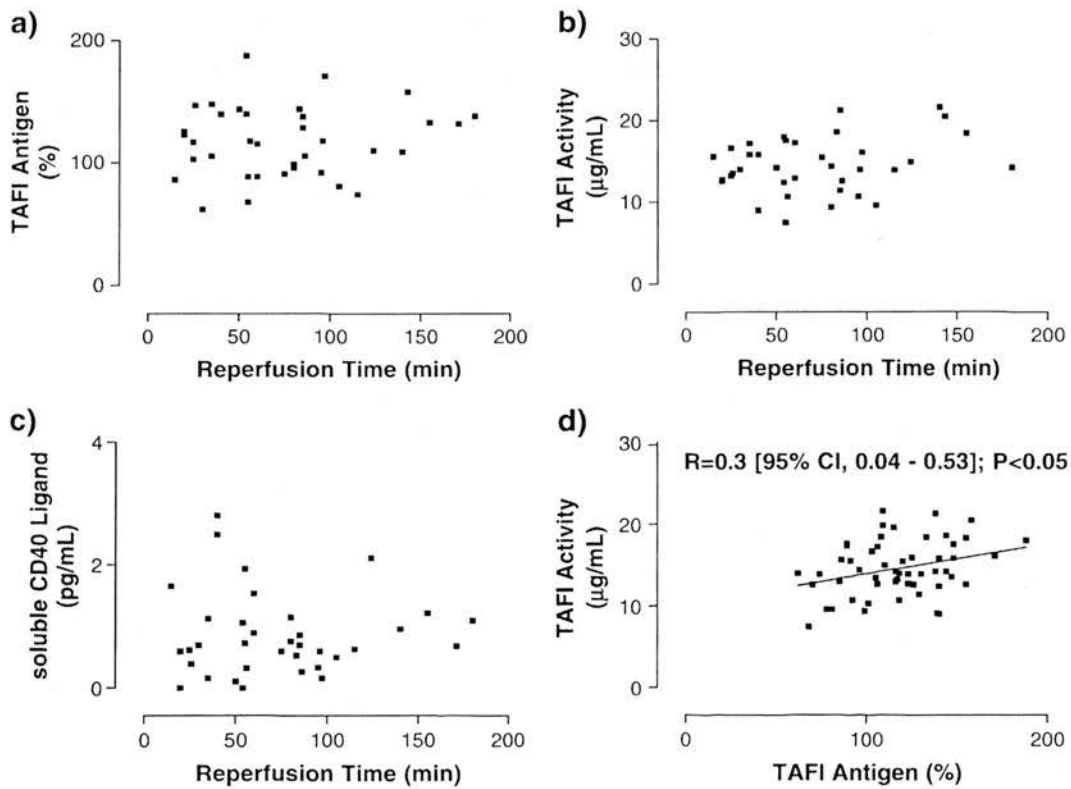
Discussion

This is the first study to examine the relationship between plasma concentrations of TAFI and sCD40L and the response to thrombolytic therapy in patients with acute ST segment elevation myocardial infarction. We found no association between coronary reperfusion and plasma concentrations of

the endogenous fibrinolytic factors, t-PA, PAI-1 and TAFI, the inflammatory marker, CRP, or the atherothrombotic mediator, sCD40L. We conclude that systemic plasma TAFI and sCD40L concentrations do not appear to influence the efficacy of therapeutic thrombolysis.

Reperfusion and creatine kinase

In keeping with previous work [28], peak creatine kinase concentrations were lower in patients who successfully reperfused following thrombolytic



**Figure 2** Comparison of time to reperfusion with plasma concentrations of (a) TAFI antigen, (b) TAFI activity, and (c) soluble CD40 ligand. (d) Relationship between plasma TAFI antigen and activity concentrations in all patients (*R* = 0.3 [95% CI: 0.04– 0.53]; *P* < 0.05).

**Table 3** Plasma concentrations of C-reactive protein and soluble CD40 ligand in patients presenting with acute ST elevation myocardial infarction

	All	Reperfusion	No reperfusion	P*	Difference* [95% CI]
CRP (mg/L)	2.0 [0.9– 5.2]	1.9 [0.8– 4.7]	2.7 [0.9– 6.5]	0.7	0.37 [–0.42 to 1.75]
CRP (mg/L; symptoms <6 h) <sup>†</sup>	1.6 [0.8– 4.0]	1.6 [0.7– 4.0]	1.9 [0.8– 4.3]	0.8	–0.02 [–0.9 to 0.7]
Soluble CD40 ligand (pg/mL) <sup>‡</sup>	0.9 ± 0.8	0.9 ± 0.7	0.9 ± 1.0	0.8	0.1 [–0.5 to 0.4]

Data are expressed as median [interquartile range] or mean ± SD. The difference between means [95% confidence interval (CI)] is also given. CRP, C-reactive protein. \*Reperfusion versus no reperfusion (unpaired *t* test or Mann–Whitney *U* test); <sup>†</sup>plasma C-reactive protein concentrations in patients presenting within 6 h of symptom onset (*n* = 87). <sup>‡</sup>(*n* = 56).

therapy. Moreover, peak creatine kinase concentrations correlated with time to reperfusion, consistent with a reduction in infarct size associated with more rapid coronary reperfusion [29].

**Endogenous fibrinolysis**

We found no association between systemic plasma t-PA or TAFI concentrations and the response to thrombolysis. These data are consistent with the observation that neither plasma t-PA nor TAFI concentrations predict reperfusion of the infarct-related artery in patients receiving thrombolytic therapy for acute stroke [30]. However, this does not exclude the possibility that local, in this case intra-coronary, activity of t-PA and TAFI may determine the stability of thrombus or sensitivity to thrombolysis. The vascular endothelium is responsible for the synthesis and release of t-PA into the circulation and when acutely stimulated, is capable of achieving local concentrations normally associated with exogenous thrombolytic therapy [31]. However, systemic plasma concentrations may not reflect local coronary vascular activity because of the substantial dilutional effect of blood passing into the systemic circulation, and the rapid removal of t-PA from the circulation by the liver (half-life ~5 min) [32]. Obtaining blood samples from the coronary sinus in patients with acute myocardial infarction prior to reperfusion therapy might provide a clearer picture of local fibrinolytic conditions but would be challenging to obtain.

Plasmin can cleave and inactivate TAFI *in vitro* [33] and it has been suggested that this mechanism may account for the reduced plasma TAFI activity and associated bleeding abnormalities observed in patients with acute promyelocytic leukaemia [34]. Local plasmin concentrations are likely to be significantly elevated following treatment with thrombolysis and this mechanism may offer an alternative explanation for our finding that systemic TAFI concentrations do not predict reperfusion.

Elevated thrombin concentrations at sites of thrombus formation are likely to favour maximal activation of local TAFI. Using the Actichrome

assay, we have measured the total amount of activatable TAFI present in plasma. However, it is not clear whether this reflects the actual concentration of TAFIa at sites of thrombus formation within the coronary circulation. In addition, we found a relatively weak correlation between TAFI antigen and activity concentrations. This may reflect differences in the stability or activity of polymorphisms of the *TAFI* gene [35].

We found no association between baseline plasma PAI-1 concentrations and the response to thrombolytic therapy in acute myocardial infarction in keeping with the findings of Paganelli et al. [36]. This is not a universal finding, however, and our data are in contrast to those of Barbash et al., who reported that plasma PAI-1 concentrations correlated with patency of the infarct-related artery [7]. This apparent disparity is likely to reflect differences in the incidence of coronary artery reocclusion due to the timing of assessment of coronary artery patency. Barbash assessed coronary artery patency at 72 h, whereas we determined reperfusion within 4 h of the administration of thrombolysis. Early reocclusion of the infarct-related coronary artery occurs in ~10% of patients receiving thrombolytic therapy for myocardial infarction [37] and has been associated with elevated PAI-1 concentrations. Indeed, in 60 patients receiving streptokinase for acute myocardial infarction, Sinkovic found that elevated baseline PAI-1 concentrations predicted coronary artery reocclusion but not reperfusion [8].

**C-reactive protein**

Although plasma CRP concentrations appeared to be lower in patients who reperfused, this was not statistically significant. In contrast, in a recent study of 319 patients receiving thrombolysis for acute myocardial infarction, elevated plasma CRP concentrations were associated with a failure to reperfuse, determined both non-invasively 2 h after admission and by coronary angiography performed within 7 days [17]. This difference may reflect the larger sample size, the greater incidence of car-

diovascular risk factors or the differing non-invasive criteria for myocardial reperfusion when compared to our study [17].

It has been suggested that elevated plasma CRP concentrations may simply reflect the extent of underlying myocardial necrosis and associated acute phase response [38]. On this basis, it would not be surprising if plasma CRP concentrations were higher in patients who fail to reperfuse. To control for this possibility we analysed the data excluding patients presenting with symptoms of greater than six hours duration, since this represents the minimum time taken for an acute increase in plasma CRP concentrations to occur. Plasma CRP concentrations were lower in this restricted population, and we again found no association with failure to reperfuse following thrombolysis.

### Soluble CD40 ligand

We found no association between plasma sCD40L concentrations and the response to thrombolytic therapy in patients with acute ST elevation myocardial infarction. These data are consistent with the observations of Andre et al. [21], which demonstrate that the instability of arterial thrombi observed in CD40 knockout mice do not appear to be due to differences in fibrin formation. The mechanism by which CD40 ligand stabilises thrombi is unclear but appears to be dependent on CD40 ligand-platelet interactions, in particular those mediated via the glycoprotein IIb/IIIa receptor [39]. The platelet glycoprotein IIb/IIIa receptor is capable of binding sCD40L directly, whilst glycoprotein IIb/IIIa receptor antagonists inhibit sCD40L release from activated platelets [40]. These data suggest a positive feedback loop whereby sCD40L stabilizes platelet–thrombus interactions under high shear rates [39]. In support of this, there was a strong correlation between platelet activation and plasma sCD40L concentrations in patients presenting with an acute coronary syndrome [22]. Given the central role of platelets in CD40 ligand-mediated signalling, it is not surprising that sCD40L concentrations predict the response to the glycoprotein IIb/IIIa inhibitor, abciximab, but not thrombolytic agents in patients with acute myocardial infarction.

### Study limitations

Although coronary angiography remains the gold standard for predicting TIMI 3 flow in the infarct-related artery, it is widely accepted that ST segment resolution is a reliable non-invasive predictor of myocardial reperfusion and prognosis following

thrombolysis [23,24,41]. We believe, therefore, that continuous ST segment monitoring is an appropriate non-invasive method with which to assess reperfusion for the purposes of this study.

Whilst there were no significant differences in the presence of major risk factors for ischemic heart disease between the reperfusion and no reperfusion groups, we acknowledge that associated comorbidity and concomitant therapies may influence both plasma concentrations of the factors measured and the success or otherwise of reperfusion therapy. However, the present study was not powered to examine the relationship between the many potential clinical variables and reperfusion.

Finally, we have examined the relationship between endogenous fibrinolytic and vascular inflammatory factors and reperfusion following thrombolysis for acute myocardial infarction. Given that elevated baseline PAI-1 concentrations predicted coronary artery reocclusion but not reperfusion [8], it remains possible that plasma concentrations of TAFI and sCD40L may influence the incidence of early re-infarction following reperfusion. Further studies are required to address this hypothesis.

### Summary

We have demonstrated that systemic plasma concentrations of TAFI, sCD40L and CRP, as well as established endogenous fibrinolytic factors, do not predict reperfusion in patients receiving thrombolytic therapy for acute ST elevation myocardial infarction. Further work is required to determine whether these findings can be extrapolated to the local endogenous fibrinolytic activity within the coronary circulation at the time of thrombotic arterial occlusion.

### Acknowledgements

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**Conflict of Interest:** This study was funded by a grant from Chest Heart and Stroke Scotland (Res02/A64). Dr Cruden performed this study whilst supported by an unrestricted educational award from Pfizer UK Ltd was not involved in any aspect of study design, data collection and interpretation, writing of the manuscript or the decision to publish. There are no other conflicts of interest.



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## **PUBLICATION 24**

# Endothelial Fibrinolytic Capacity Predicts Future Adverse Cardiovascular Events in Patients With Coronary Heart Disease

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**Objective**—The endothelium-derived fibrinolytic factor tissue plasminogen activator (t-PA) is a major determinant of vessel patency after coronary plaque rupture and thrombosis. We assessed whether endothelial fibrinolytic capacity predicts atherothrombotic events in patients with coronary heart disease.

**Methods and Results**—Plasma t-PA and plasminogen activator inhibitor (PAI)-1 concentrations were measured during intrabrachial substance P infusion in 98 patients with angiographically proven stable coronary heart disease. Forearm blood flow was measured during infusion of substance P and sodium nitroprusside. Cardiovascular events (cardiovascular death, myocardial infarction [MI], ischemic stroke [CVA], and emergency hospitalization for unstable angina) were determined during 42 months of follow-up. Patients experiencing a cardiovascular event ( $n=19$ ) had similar baseline characteristics to those free of events. Substance P caused a dose-dependent increase in plasma t-PA concentrations ( $P<0.001$ ). However, net t-PA release was 72% lower in the patients who experienced death, MI, or CVA, and 48% lower in those who suffered death, MI, CVA or hospitalization for unstable angina ( $P<0.05$ ). Major adverse cardiovascular events were most frequent in those with the lowest fibrinolytic capacity ( $P=0.03$  for trend); patients with the lowest quartile of t-PA release had the highest rate of adverse events ( $P=0.01$ ).

**Conclusion**—Endothelial fibrinolytic capacity, as measured by stimulated t-PA release, predicts the future risk of adverse cardiovascular events in patients with coronary heart disease. We suggest that endothelial fibrinolytic capacity is a powerful novel determinant of cardiovascular risk. (*Arterioscler Thromb Vasc Biol.* 2007;27:1651-1656.)

**Key Words:** coronary heart disease ■ endothelium ■ fibrinolysis ■ survival ■ vasodilation

The endogenous fibrinolytic system protects the circulation from intravascular fibrin formation and thrombosis. In the presence of developing thrombus, the fibrinolytic factor tissue plasminogen activator (t-PA) is rapidly released from the vascular endothelium by the coagulation factors thrombin and factor Xa<sup>1</sup> and causes a 1000-fold increase in the enzymatic conversion of plasminogen to plasmin.<sup>2,3</sup> This ensures that rapid plasmin generation, fibrin degradation, and clot dissolution are tightly regulated and localized to sites of vascular injury and thrombus formation. Thus, the rapidity and extent of acute t-PA release from the endothelium is a critical factor in determining the efficacy of local endogenous fibrinolysis.<sup>4,5</sup>

Areas of endothelial denudation and thrombus deposition are a common finding on the surface of atheromatous plaques and are often subclinical.<sup>6</sup> Through t-PA release, endogenous fibrinolysis is usually able to prevent thrombus propagation, vessel occlusion, and tissue infarction, although organization of the residual thrombus may lead to plaque growth and expansion.<sup>6</sup> The resolution of thrombus after atheromatous

plaque rupture, and the resulting clinical sequelae, may therefore be critically dependent on the efficacy on endogenous fibrinolysis.<sup>7</sup> Accordingly the capacity of the endothelium to release t-PA may predict the outcome of individual plaque events and long-term cardiovascular risk.

Intraarterial infusion of substance P<sup>8</sup> and bradykinin<sup>9</sup> cause a rapid and sustained release of t-PA from the endothelium, and acute stimulated t-PA release has been measured within the forearm<sup>8,10</sup> and coronary<sup>11,12</sup> circulations of man. Previous studies have demonstrated impaired forearm t-PA release in cigarette smokers<sup>13</sup> and patients with hypertension<sup>14</sup> who are at particular risk of coronary thrombosis and myocardial infarction (MI).<sup>15,16</sup> Although this suggests that endogenous fibrinolysis plays an important role in the pathogenesis of coronary thrombosis, the relationship between the capacity to release t-PA and the future risk of adverse cardiovascular events is unknown. The aim of the present study was, therefore, to determine whether endothelial fibrinolytic capacity predicts the future risk of atherothrombotic events in patients with stable coronary heart disease (CHD).

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## Methods

### Patients

We recruited patients with angiographically proven CHD defined as  $\geq 50\%$  luminal stenosis of at least one major epicardial coronary vessel. All patients had stable anginal symptoms and had not undergone coronary revascularization within the preceding 3 months. Exclusion criteria were significant cardiac failure, renal impairment, systolic blood pressure  $<100$  or  $>190$  mm Hg, or diabetes mellitus. All studies were undertaken with the approval of the local Research Ethics Committee, the written informed consent of each subject, and in accordance with the Declaration of Helsinki.

### Venous Sampling and Assays

Venous blood was collected into tubes containing gel, ethylene diamine tetra-acetic acid, acidified buffered citrate, and trisodium citrate. Platelet-free plasma and serum were stored at  $-80^{\circ}\text{C}$  before assay. Plasma t-PA and PAI type 1 (PAI-1) antigen concentrations (Coalixa, Chromogenix) were determined using enzyme-linked immunosorbent assays.<sup>8,13</sup> Highly sensitive assays of C-reactive protein (hs-CRP) were undertaken on serum using the method of particle-enhanced immunonephelometry (Behring BN II nephelometer). Serum biochemical analysis and hematocrit estimations were undertaken by the hospital Clinical Biochemistry and Hematology Laboratories, respectively.

### Forearm Study Protocol

Subjects abstained from alcohol for 24 hours and from food, tobacco, and caffeine-containing drinks for at least 4 hours before each study visit. Cardioactive medications were withheld on the morning of each study. All studies were carried out in a quiet temperature controlled room maintained at  $22$  to  $25^{\circ}\text{C}$ . Patients rested recumbent, strain gauges and cuffs were applied. A 17-G venous cannula was inserted into the antecubital vein of each arm and the brachial artery of the nondominant arm was cannulated with a 27-G needle (Cooper's Needle Works Ltd). Forearm blood flow (FBF) was measured in both forearms by venous occlusion plethysmography using mercury-in-silastic strain gauges as previously described.<sup>8,13</sup> Blood pressure and heart rate were measured using a semiautomated noninvasive sphygmomanometer.

After a 30-minute saline infusion, intraarterial substance P (Clnalfa) at 2, 4, and 8 pmol/min (endothelium-dependent vasodilator releasing t-PA<sup>8</sup>) and sodium nitroprusside (David Bull Laboratories) at 2, 4, and 8  $\mu\text{g}/\text{min}$  (endothelium-independent vasodilator which does not release t-PA<sup>8,17</sup>) were infused intraarterially for 6 minutes at each dose. The vasodilators were administered in a randomized order with a 15-minute saline washout period between each agent. Forearm blood flow was measured at baseline and with each dose of vasodilator. Venous blood sampling for plasma t-PA and PAI-1 antigen concentration estimation was undertaken before and after each 6-minute infusion period of substance P doses 2, 4, and 8 pmol/min.

### Long-Term Follow-Up

Atherothrombotic cardiovascular events, defined as death from a cardiovascular cause, MI, ischemic stroke (CVA), and emergency hospitalization for unstable angina, were determined through the Information and Statistics Division of the National Health Service in Scotland and the General Register Office in Scotland. These databases combine high quality data and consistency, with national coverage and the ability to link data to allow patient based analysis and follow-up. These systems have been successfully applied to evaluate cardiovascular morbidity and mortality for subjects resident within Scotland.<sup>18</sup> Diagnostic criteria for acute myocardial infarction and unstable angina were as previously validated in the GRACE (Global Registry of Acute Coronary Events) registry,<sup>19</sup> a prospective multi-center observational study of patients with the full spectrum of acute coronary syndromes (full criteria shown in data supplement online, please see <http://atvb.ahajournals.org>). Patients with unstable

symptoms who were hospitalized for  $<1$  day and patients with perioperative associated acute myocardial infarction were excluded.

### Statistical Analysis

Data of baseline characteristics are expressed as mean  $\pm$  SD or frequency (n, %) and compared using 2-tailed Student *t* test and Fisher exact test where appropriate. Responses to substance P and sodium nitroprusside were examined by analysis of variance (ANOVA) for repeated measures. Forearm blood flow responses to the vasodilators were analyzed as the ratio between the infused and control arms expressed as a percentage of the ratio measured during the baseline control period.<sup>20</sup> Estimated net release of t-PA was defined previously as the product of the infused forearm plasma flow, and the concentration difference between the infused and noninfused forearms.<sup>5,8,13</sup> Cumulative event rates were estimated by Kaplan-Meier survival curves, and probability values were determined with the log-rank test. In those patients who experienced multiple events, survival analysis was restricted to the first event. For Kaplan-Meier analyses, subjects were divided into quartiles according to estimated net release of t-PA antigen. All statistical analysis was completed using GraphPad Prism (GraphPad Software) with statistical significance being assigned at the 5% level.

## Results

Endothelial fibrinolytic capacity was measured in 98 subjects with angiographically proven CHD who were followed up for a median of 42 months (range 5 to 51 months). Over the follow-up period, 2 patients died from cardiovascular disease, 2 suffered a MI, 2 had an ischemic CVA, and 13 had an emergency admission for unstable angina. In general, patients experiencing cardiovascular events had similar baseline characteristics and use of secondary preventative medications to those free from events (Table), although plasma glucose levels were slightly higher in those subsequently experiencing clinical events ( $6.1 \pm 0.9$  versus  $5.6 \pm 0.8$  mmol/L, respectively,  $P=0.03$ ).

Substance P caused a dose-dependent increase in plasma t-PA ( $P<0.001$ , ANOVA) but not PAI-1 ( $P=\text{ns}$ ) concentrations. The concentration differences of t-PA antigen between the forearms and estimated net release of t-PA both increased dose dependently ( $P<0.001$  for both, ANOVA) although this increase was reduced in subjects who subsequently experienced cardiovascular events ( $P<0.05$ , ANOVA; Figure 1). Specifically, net release of t-PA during substance P infusion was 72% lower in those subjects suffering cardiovascular death, MI, or CVA ( $P=0.02$ , ANOVA), and 48% lower in those with cardiovascular death, MI or CVA, or hospitalization for unstable angina ( $P=0.008$ , ANOVA). Major adverse cardiovascular events were most frequent in those with the lowest fibrinolytic capacity ( $P=0.03$  for trend) such that patients with the lowest quartile of t-PA release had the highest rate of adverse events ( $P=0.01$ ; Figure 2).

Although baseline parameters were similarly distributed between subjects with and without events, baseline plasma PAI-1 antigen and female gender were positively correlated with t-PA release ( $P<0.05$ ), whereas cigarette smoking was associated with a reduction in substance P-induced t-PA release ( $P=0.05$ ). There was no association between acute t-PA release and plasma glucose concentrations ( $159 \pm 24$  versus  $168 \pm 26$ ,  $P=0.80$ ; area under the curve for t-PA release in subjects  $\leq$  versus  $>$  median glucose concentration).

**Baseline Subject Characteristics**

	All Patients (n=98)	Subjects With Events (n=19)	Subjects Without Events (n=79)	P Value*
Age, years	59±7	62±8	59±7	0.10
Sex, male/female	80/18	17/2	63/16	0.51
BMI, kg/m <sup>2</sup>	29±4	28±4	29±5	0.27
Systolic BP, mm Hg	134±18	138±20	133±17	0.26
Diastolic BP, mm Hg	75±10	77±10	74±10	0.71
Pulse, bpm	57±9	56±8	57±9	0.70
Cigarette smoker, %	31	32	30	1.00
Co-morbidity, %				
Previous MI	42	53	39	0.31
Hypertension	48	47	48	1.00
FHx CHD	33	47	29	0.17
Symptomatic CVD	4	11	3	0.17
Extent of coronary disease, %				
1 vessel	46	47	46	0.73
2 vessels	26	26	26	0.84
3 vessels	28	26	28	0.98
Medical therapy, %				
Aspirin	99	100	99	0.62
Lipid lowering therapy	93	95	92	0.72
ACE inhibitor, ARB	31	37	29	0.51
Urea, mmol/L	5.6±1.3	5.3±1.4	5.6±1.3	0.41
Creatinine, µmol/L	94±13	96±16	93±12	0.29
Glucose, mmol/L	5.7±0.9	6.1±0.9	5.6±0.8	0.03*
Total chol, mmol/L	4.5±1.0	4.5±1.1	4.5±0.9	0.88
LDL chol, mmol/L	2.5±0.8	2.5±1.1	2.5±0.6	0.95
t-PA antigen, ng/mL	9.7±3.9	10.1±5.0	9.6±3.6	0.62
PAI-1 antigen, ng/mL	43.7±24.5	36.3±17.9	45.4±25.6	0.16
C-reactive protein, mg/L	1.91±1.87	1.98±1.84	1.90±1.84	0.92

Values are mean±SD.

\*Unpaired *t* test or Fishers exact test for subjects with, vs without, events (death from a cardiovascular cause, MI, ischemic CVA, hospitalisation for unstable angina). As C-reactive protein values were not normally distributed they were log transformed before statistical analysis.

BMI indicates body mass index; bpm, beats per minute; MI, myocardial infarction; CHD, coronary heart disease; FHx, family history; CVD, cerebrovascular disease; ACE, angiotensin converting enzyme; ARB, angiotensin receptor blocker; chol, cholesterol; t-PA, tissue plasminogen activator; PAI-1, plasminogen activator inhibitor-1.

There was no difference in resting forearm blood flow between those subjects with and without atherothrombotic events ( $2.9 \pm 0.3$  mL/100 mL tissue/min versus  $2.8 \pm 0.3$  mL/100 mL tissue/min, respectively;  $P=0.37$ ). Substance P and sodium nitroprusside caused dose-dependent increases in infused forearm blood flow in all subjects ( $P<0.001$ , ANOVA). There were no differences in either endothelium-dependent or -independent blood flow responses between subjects in either group (Figure 3).

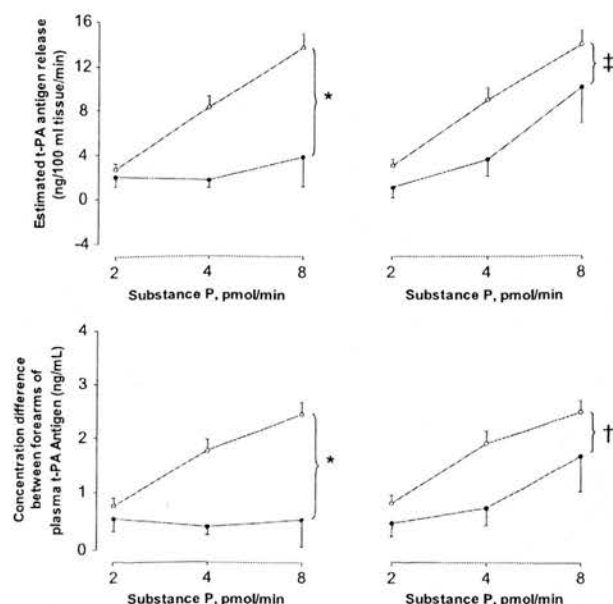
### Discussion

This is the first study to assess the relationship between endothelial fibrinolytic capacity and the future risk of atherothrombotic cardiovascular events. The population that we chose to study was a homogenous cohort of patients with

stable CHD and a typical distribution of conventional cardiovascular risk factors. Even though this was a relatively low risk population receiving optimal secondary preventative therapy, endothelial fibrinolytic capacity has emerged as a powerful novel determinant of future cardiovascular risk.

In a metaanalysis of prospective observational studies,<sup>21</sup> the risk of CHD is increased in subjects with plasma t-PA antigen concentrations in the highest tertile compared with those in the lowest tertile. This may seem counterintuitive but in part reflects the concomitant rise of plasma PAI-1 concentrations and the associated reduction in t-PA activity. Moreover, in the basal state, the endogenous fibrinolysis system is effectively inactive and does not influence in situ thrombus formation. Neither basal plasma t-PA nor PAI-1 concentrations control the local vascular fibrinolytic capacity, which is

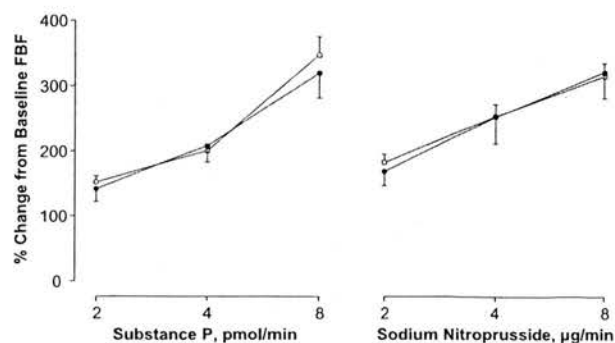




**Figure 1.** Concentration differences between the forearms of plasma tissue plasminogen activator (t-PA) antigen (lower panels) and estimated net release of t-PA antigen (upper panels) in subjects with (●), and without (○), death, MI, or CVA (left hand panels) or death, MI, CVA, or hospitalization for unstable angina (right hand panels). \* $P=0.02$ , ANOVA (●) vs (○); † $P=0.004$ , ANOVA (●) vs (○); ‡ $P=0.008$ , ANOVA (●) vs (○).

determined by the acute release of t-PA from the endothelium.<sup>4</sup> This underscores the importance of assessing the pathophysiologically relevant measure of acute stimulated endothelial t-PA release.

The positive association between forearm t-PA release and female gender is in keeping with previous reports.<sup>22,23</sup> Furthermore, we and others have also previously demonstrated that cigarette smoking<sup>11,13,24</sup> but not hypercholesterolemia<sup>25</sup> is associated with a characteristic and substantial reduction in endothelial t-PA release. There is a good correlation and consistency between the endothelial fibrinolytic capacity of the forearm<sup>13,26</sup> circulation and the coronary vascular bed.<sup>11,12</sup> Although the forearm vascular bed is relatively protected from the development of atheroma, it therefore seems likely that changes in its fibrinolytic capacity are indicative of a systemic effect and not simply local plaque burden. These

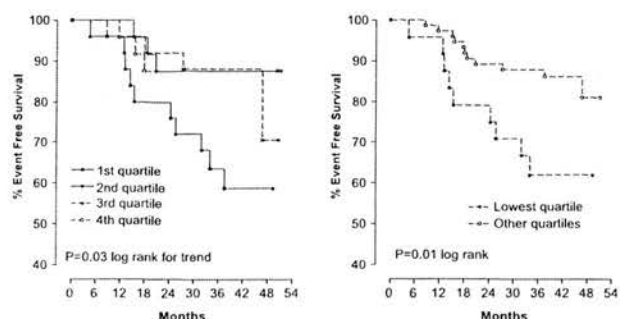


**Figure 3.** Forearm blood flow (FBF) during incremental doses of substance P (left), sodium nitroprusside (right) in subjects with (●) and without (○) death, MI, CVA, or hospitalization for unstable angina.  $P<0.001$ , ANOVA dose response;  $P=ns$ , (●) vs (○), for both vasodilators.

considerations are, therefore, in keeping with the hypothesis that atherosclerosis is a systemic disorder, and that acute t-PA release should be considered a distinct marker of endothelial function.

Intravascular thrombus formation is a key feature of clinical atherosclerotic events associated with eroded or unstable coronary plaques. The importance of endogenous t-PA release is exemplified by the high rate of spontaneous reperfusion in the infarct related artery after acute myocardial infarction, occurring in up to 30% of patients within the first 12 hours.<sup>27–29</sup> Any reduction in the acute dynamic fibrinolytic response decreases the capacity to lyse intraluminal thrombus and the likelihood of restoring vessel patency. In this prospective observational cohort study, we have further demonstrated that the capacity to release t-PA appears to be a major determinant of the risk of cardiovascular events and suggests that endothelial fibrinolytic capacity has a crucial role in the pathogenesis of atherothrombosis.

Endothelial dysfunction is characterized by the disruption of multiple homeostatic pathways predisposing to vasoconstriction, platelet activation, and thrombosis. To date, most clinical studies on endothelial function have focused on endothelium-dependent vasomotion with decreased responses associated with an increased incidence of future adverse events.<sup>30–32</sup> Although a useful surrogate marker, the pathophysiological mechanism linking impaired endothelium-dependent vascular smooth muscle relaxation and future atherothrombotic events remains unclear. Moreover, the regulation of vessel tone may not be the facet of vascular function most closely allied to the future risk of atherothrombotic events. In this current study, we did not observe a difference in endothelium-dependent or -independent blood flow responses between subjects with, and without, subsequent cardiovascular events. This disparity may reflect a lack of power or the use of differing endothelium-dependent vasodilators. Thus far, studies assessing the prognostic value of endothelium-dependent vasodilatation have used acetylcholine. We chose to use substance P because this is a potent



**Figure 2.** Cumulative proportion of patients without cardiovascular events during follow-up (Kaplan-Meier). Subjects are divided into quartiles for estimated net release of t-PA antigen.  $P=0.03$ , log rank for trend;  $P=0.01$ , lowest vs upper quartiles.

stimulant of endothelial t-PA release, and acetylcholine does not cause demonstrable t-PA release.<sup>9</sup> Reports of preserved endothelium-dependent vasodilatation in smokers<sup>33</sup> and in patients with hypertension<sup>34</sup> despite reduced acute t-PA release<sup>24,35</sup> suggest that, in some circumstances, reduced t-PA release may be a more sensitive marker of endothelial dysfunction.<sup>5</sup> These data also highlight the complexity of vascular biology and demonstrate that endothelial dysfunction is not a single clinical entity encompassing a uniform pathophysiological response to vascular injury.

### Study Limitations

Although we cannot determine basal t-PA release using the venovenous technique described,<sup>5</sup> calculation of net t-PA release provides an accurate assessment of stimulated t-PA release with good reproducibility<sup>25</sup> and basal release contributes only a small proportion of the overall venous plasma t-PA concentration. Furthermore, previous work has demonstrated that basal plasma t-PA concentrations do not control the local vascular fibrinolytic capacity which is in fact determined by the acute release of t-PA from the endothelium.<sup>4</sup>

Plaque growth is induced by episodic subclinical plaque disruption<sup>6</sup> and if local t-PA release is impaired, the continued presence of thrombus may favor smooth muscle migration, the production of new connective tissue, and plaque expansion.<sup>36</sup> In keeping with this hypothesis, genetic murine models of plasminogen deficiency<sup>37,38</sup> as well as PAI-1 overexpression<sup>39</sup> have shown that reduced fibrinolytic potential is associated with enhanced macrovascular fibrin deposition and accelerated atherogenesis. As we do not have follow-up angiographic data, we do not know whether those subjects with the lowest local t-PA release or recurrent cardiovascular events exhibited a greater progression of angiographic disease over the study period. We have previously demonstrated an inverse correlation between acute coronary t-PA release and local atheromatous plaque burden,<sup>11</sup> and prospective studies of coronary t-PA release and quantification of disease progression would be of interest.

Comparable numbers of patients in each group were receiving secondary preventative medications including ACE inhibitors. Although treatment with ACE inhibitors enhances bradykinin-mediated t-PA release,<sup>40,41</sup> these do not influence t-PA release in response to infusion of substance P.<sup>40</sup> In keeping with the risk factor profile and use of secondary preventative therapies in our study cohort, relatively few events occurred over the study period. Despite this, similar results were noted for outcomes regardless of whether hospitalization for unstable angina was included. Although the risk factor profile and severity of coronary artery disease were similar in those patients with or without events during follow-up, the relatively modest study numbers and follow-up period mean we were unable to explore in detail potential interactions and independence of endothelial fibrinolytic capacity with conventional cardiovascular risk factors or the extent of coronary artery disease. Indeed, given the modest number of subjects and clinical end points, our results should be interpreted with caution. It would be desirable to increase subject numbers and clinical end points further, but the

invasive nature and complexity of undertaking such detailed physiological studies presents many challenges when attempting to apply this technique to larger population-based clinical studies.

### Conclusion

t-PA release from the endothelium is a distinct marker of endothelial function. In patients with stable CHD, we have shown that a reduction in acute t-PA release predicts an increased risk of adverse cardiovascular events. Further studies of the factors modifying the endogenous fibrinolytic capacity have the potential to provide major new insights into the pathophysiology of CHD and to shape future therapeutic interventions.

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### Disclosures

None.

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## Ischemic and Thrombotic Effects of Dilute Diesel-Exhaust Inhalation in Men with Coronary Heart Disease

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### ABSTRACT

#### BACKGROUND

Exposure to air pollution from traffic is associated with adverse cardiovascular events. The mechanisms for this association are unknown. We conducted a controlled exposure to dilute diesel exhaust in patients with stable coronary heart disease to determine the direct effect of air pollution on myocardial, vascular, and fibrinolytic function.

#### METHODS

In a double-blind, randomized, crossover study, 20 men with prior myocardial infarction were exposed, in two separate sessions, to dilute diesel exhaust (300  $\mu$ g per cubic meter) or filtered air for 1 hour during periods of rest and moderate exercise in a controlled-exposure facility. During the exposure, myocardial ischemia was quantified by ST-segment analysis using continuous 12-lead electrocardiography. Six hours after exposure, vasomotor and fibrinolytic function were assessed by means of intraarterial agonist infusions.

#### RESULTS

During both exposure sessions, the heart rate increased with exercise ( $P < 0.001$ ); the increase was similar during exposure to diesel exhaust and exposure to filtered air ( $P = 0.67$ ). Exercise-induced ST-segment depression was present in all patients, but there was a greater increase in the ischemic burden during exposure to diesel exhaust ( $-22 \pm 4$  vs.  $-8 \pm 6$  millivolt seconds,  $P < 0.001$ ). Exposure to diesel exhaust did not aggravate preexisting vasomotor dysfunction, but it did reduce the acute release of endothelial tissue plasminogen activator ( $P = 0.009$ ; 35% decrease in the area under the curve).

#### CONCLUSIONS

Brief exposure to dilute diesel exhaust promotes myocardial ischemia and inhibits endogenous fibrinolytic capacity in men with stable coronary heart disease. Our findings point to ischemic and thrombotic mechanisms that may explain in part the observation that exposure to combustion-derived air pollution is associated with adverse cardiovascular events. (ClinicalTrials.gov number, NCT00437138.)

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THE WORLD HEALTH ORGANIZATION (WHO) estimates that air pollution is responsible for 800,000 premature deaths worldwide each year.<sup>1</sup> Short-term exposure to air pollution has been associated with increases in cardiovascular morbidity and mortality, with deaths due to ischemia, arrhythmia, and heart failure.<sup>2</sup> In a large cohort study from the United States, Miller et al. recently reported that long-term exposure to air pollution increases the risk of death from cardiovascular disease by 76%.<sup>3</sup> These associations are strongest for fine particulate air pollutants (particulate matter of less than 2.5  $\mu\text{m}$  in aerodynamic diameter [ $\text{PM}_{2.5}$ ]), of which the combustion-derived nanoparticulate in diesel exhaust is an important component.<sup>4</sup> Substantial improvements in air quality have occurred in the developed world over the past 50 years, yet the association between  $\text{PM}_{2.5}$  and mortality has no apparent threshold and is evident below current air-quality standards.<sup>5</sup>

Preclinical models of exposure to particulate air pollution demonstrate accelerated atherosclerotic plaque development<sup>6</sup> and increased *in vitro*<sup>7</sup> and *in vivo*<sup>8</sup> platelet aggregation. Epidemiologic and observational clinical studies suggest that exposure to air pollution may worsen symptoms of angina,<sup>9</sup> exacerbate exercise-induced myocardial ischemia,<sup>10,11</sup> and trigger acute myocardial infarction.<sup>12,13</sup> These clinical findings are limited by imprecision in the measurement of pollution exposure, the effect of potential confounding environmental and social factors, and the lack of mechanistic data.<sup>14</sup> Controlled exposures to air pollutants can help address these shortcomings by providing a precisely defined exposure in a regulated environment that facilitates investigation with validated biomarkers and surrogate measures of cardiovascular health. Using a carefully characterized exposure system, we have previously shown that exposure to dilute diesel exhaust in healthy volunteers causes lung inflammation,<sup>15</sup> depletion of airway antioxidant defenses,<sup>16</sup> and impairment of vascular and fibrinolytic function.<sup>17</sup>

To our knowledge, there have been no controlled exposures in patients with coronary heart disease, an important population that may be particularly susceptible to the adverse cardiovascular effects of air pollution. We assessed the effect of inhalation of dilute diesel exhaust on myocardial, vascular, and fibrinolytic function in a population of patients with stable coronary heart disease.

## METHODS

### SUBJECTS

Twenty men with stable coronary artery disease participated in this study, which was performed with the approval of the local research ethics committee, in accordance with the Declaration of Helsinki, and with the written informed consent of all participants.

All the men had proven coronary heart disease, with a previous myocardial infarction (>6 months before enrollment) treated by primary angioplasty and stenting, and were receiving standard secondary preventive therapy. Men with angina pectoris (Canadian Cardiovascular Society class  $\geq 2$ ), a history of arrhythmia, diabetes mellitus, uncontrolled hypertension, or renal or hepatic failure, as well as those with unstable coronary disease (acute coronary syndrome or symptoms of instability 3 months before enrollment), were excluded. All eligible volunteers were invited to a prestudy screening for exercise stress testing; subjects who were unable to achieve stage 2 of the Bruce protocol or who had marked changes on an electrocardiogram (left bundle-branch block, early ST-segment depression >2 mm) and those in whom hypotension developed were excluded. Current smokers and men with asthma, substantial occupational exposure to air pollution, or an intercurrent illness were also excluded from the study.

### STUDY DESIGN

Using a randomized, double-blind, crossover study design, we evaluated the subjects in two 8 a.m. sessions at least 2 weeks apart. In each session, the subjects were exposed to controlled amounts of dilute diesel exhaust or filtered air. Each subject was exposed for 1 hour in an exposure chamber, as previously described.<sup>15</sup> During each exposure, the subjects performed two 15-minute periods of exercise on a bicycle ergometer separated by two 15-minute periods of rest. For each subject, the ergometer workload was calibrated to achieve a ventilation of 15 liters per minute per square meter of body-surface area to ensure a similar exposure on both occasions. The workload was constant for both exposures and was equivalent to stage 2 of the Bruce protocol (range, 110 to 150 watts; 5 to 7 metabolic equivalents). All subjects were fitted with 12-lead Holter electrocardiographic monitors (Medical Lifecard 12 Digital Holter Recorder, Del Mar Reynolds). In accordance with

previous exposure studies in healthy volunteers, vascular assessments were made 6 to 8 hours after exposure to diesel exhaust or filtered air.<sup>17</sup>

#### DIESEL-EXHAUST EXPOSURE

The diesel exhaust was generated from an idling Volvo diesel engine (Volvo TD45, 4.5 liters, 4 cylinders, 680 rpm) from low-sulfur gas-oil E10 (Preem), as described previously.<sup>15</sup> More than 90% of the exhaust was shunted away, and the remainder diluted with filtered air heated to 20°C (relative humidity approximately 50%) before being fed into a whole-body exposure chamber (3.0 m by 3.0 m by 2.4 m) at a steady-state concentration.

The chamber was monitored continuously for pollutants, with exposures standardized with the use of nitrogen oxide concentrations to deliver a particulate matter concentration of 300  $\mu\text{g}$  per cubic meter (median particle diameter, 54 nm; range, 20 to 120). There was little variation between exposures in the mean ( $\pm\text{SE}$ ) number of particles ( $1.26\pm0.01\times10^6$  particles per cubic centimeter) or in the concentrations of nitrogen oxide ( $4.45\pm0.02$  ppm), nitrogen dioxide ( $1.01\pm0.01$  ppm), nitric oxide ( $3.45\pm0.03$  ppm), carbon monoxide ( $2.9\pm0.1$  ppm), and total hydrocarbon ( $2.8\pm0.1$  ppm). The predominant polycyclic aromatic hydrocarbons (approximately 90% of the total) were phenanthrene, fluorene, 2-methylfluorene, dibenzothiophene, and different methyl-substituted phenanthrenes. Only a minor fraction of polycyclic aromatic hydrocarbons (3.5%) was associated with particulate matter: 0.04% total particulate matter and 0.06% particulate-matter organic fraction. The concentration of particulate matter of less than 10  $\mu\text{m}$  in aerodynamic diameter ( $\text{PM}_{10}$ ) in the exposure chamber exceeded the WHO air-quality standard of 50  $\mu\text{g}$  per cubic meter by a factor of 6, and the nitrogen dioxide concentration exceeded the WHO standard of 0.105 ppm by a factor of 10.<sup>18</sup>

#### VASCULAR STUDY

All subjects underwent brachial-artery cannulation with a 27-standard wire-gauge steel needle. After a 30-minute baseline saline infusion, subjects were given infusions of acetylcholine at rates of 5, 10, and 20  $\mu\text{g}$  per minute (endothelium-dependent vasodilator, Clinalfa), bradykinin at rates of 100, 300, and 1000 pmol per minute (endothelium-dependent vasodilator that releases tissue plasminogen activator [t-PA], Clinalfa), and sodium nitroprusside at rates of 2, 4, and 8  $\mu\text{g}$  per minute

Table 1. Baseline Characteristics of the 20 Subjects with Coronary Heart Disease.\*

Characteristic	Value
Age (yr)	60 $\pm$ 1
Smoking history (no. of subjects)	
Nonsmoker	12
Former smoker	8
Current smoker	0
Hypertension (no. of subjects)	8
Height (cm)	173 $\pm$ 6
Weight (kg)	79 $\pm$ 3
Body-mass index	27 $\pm$ 1
Time since index infarction (mo)	35 $\pm$ 4
Coronary angiographic findings	
No. of diseased vessels	
1	13
2	6
3	1
Culprit lesion (no. of subjects)	
Left anterior descending coronary artery	14
Circumflex coronary artery	4
Right coronary artery	2
Cholesterol (mg/dl)	
Total	173 $\pm$ 6
LDL	100 $\pm$ 8
HDL	48 $\pm$ 2
Triglycerides (mg/dl)	128 $\pm$ 23
Fasting glucose (mg/dl)	102 $\pm$ 6
Medications (no. of subjects)	
Aspirin	20
Statin	18
Beta-blocker	15
ACE inhibitor or angiotensin-receptor blocker†	4

\* Plus-minus values are means  $\pm\text{SE}$ . The body-mass index is the weight in kilograms divided by the square of the height in meters. LDL denotes low-density lipoprotein, HDL high-density lipoprotein, and ACE angiotensin-converting enzyme. To convert the values for cholesterol to millimoles per liter, multiply by 0.02586. To convert the values for triglycerides to millimoles per liter, multiply by 0.01129. To convert the values for glucose to millimoles per liter, multiply by 0.05551.

† ACE inhibitor therapy was withdrawn 7 days before each vascular study. All other regular medications were continued throughout the study.

(endothelium-independent vasodilator, David Bull Laboratories); each infusion was given for 6 minutes. Infusions of the three vasodilators were separated by 20-minute saline infusions and given in a randomized order. Therapy with angiotensin-converting-enzyme inhibitors was withdrawn

Table 2. Effect of Exercise on Heart Rate and ST Segment in the 20 Subjects during Exposures to Filtered Air and Diesel Exhaust.\*

Characteristic	Filtered Air	Diesel Exhaust	P Value†
<b>Exercise phase 1</b>			
Heart rate — bpm			
Baseline	63±2	61±2	0.24
Maximum	87±3	86±3	0.67
Maximum ST-segment change (μV)			
Lead II	-28±13	-56±10	0.03
Lead V <sub>2</sub>	-28±10	-41±12	0.18
Lead V <sub>5</sub>	-14±8	-33±9	0.04
Change in ischemic burden (mVsec)			
Lead II	-11±5	-23±4	0.004
Lead V <sub>2</sub>	-13±5	-21±6	0.04
Lead V <sub>5</sub>	-4±3	-12±4	0.01
<b>Exercise phase 2</b>			
Heart rate (bpm)			
Baseline	67±2	65±2	0.35
Maximum	91±3	87±3	0.12
Maximum ST-segment change (μV)			
Lead II	-17±15	-49±12	0.006
Lead V <sub>2</sub>	-18±12	-41±13	0.04
Lead V <sub>5</sub>	-7±9	-28±10	0.02
Change in ischemic burden (mVsec)			
Lead II	-8±6	-22±4	0.0007
Lead V <sub>2</sub>	-11±5	-20±6	0.02
Lead V <sub>5</sub>	-2±3	-12±5	0.006

\* Plus-minus values are means ±SE; mVsec denotes millivolt seconds.

† P values were calculated with Student's t-test.

7 days before each vascular study, because it augments bradykinin-induced release of endothelial t-PA.<sup>19</sup> All other medications were continued throughout the study.

Forearm blood flow was measured in both arms by venous occlusion plethysmography with the use of mercury-in-Silastic strain gauges, as described previously.<sup>20</sup> Heart rate and blood pressure in the noninfused arm were monitored at intervals throughout each study while the subject was in the supine position, with the use of a semi-automated, noninvasive oscillometric sphygmomanometer.

#### FIBRINOLYTIC AND INFLAMMATORY MARKERS

Blood (10 ml) was withdrawn into acidified buffered citrate (Stabilyte tubes, Biopool International)

for t-PA assays and into citrate (BD Vacutainer) for plasminogen activator inhibitor type 1 (PAI-1) assays. Plasma t-PA and PAI-1 antigen concentrations were determined by means of enzyme-linked immunosorbent assays (TintElize t-PA, Biopool EIA; Coaliza PAI-1; and Chromogenix AB). Serum C-reactive protein concentrations were measured with an immunonephelometric assay (BN II nephelometer, Dade Behring).

#### DATA ANALYSIS

Electrocardiographic recordings were analyzed with the use of the Medical Pathfinder Digital 700 Series Analysis System (Del Mar Reynolds). ST-segment deviation was calculated by comparing the ST segment during each 15-minute exercise test with the average ST segment for the 15-minute period immediately before the start of the exposure. The ST-segment amplitude was determined at the J point plus 80 msec. The ischemic burden during each exercise test was calculated as the product of the change in ST-segment amplitude and the duration of exercise. Leads II, V<sub>2</sub>, and V<sub>5</sub> were selected a priori for ST-segment analysis to reflect separate regions of myocardium. The maximum ST-segment depression and ischemic burden were determined for these leads individually and as a composite.

Plethysmographic data and net t-PA release were determined as described previously.<sup>20,21</sup>

#### STATISTICAL ANALYSIS

Continuous variables are reported as means ±SE. Analysis of variance with repeated measures and a two-tailed Student's t-test were performed as appropriate with the use of GraphPad Prism software. A two-sided P value of less than 0.05 was considered to indicate statistical significance.

## RESULTS

Subjects were all middle-aged men with predominantly single-vessel coronary artery disease (Table 1). They reported no symptoms of angina and had no major arrhythmias during exposure or in the subsequent 24 hours.

#### MYOCARDIAL ISCHEMIA

The heart rate increased with exercise during exposures to diesel exhaust and filtered air ( $P<0.001$  for both comparisons with the baseline rates;  $P=0.67$  for the comparison of rates during exposure to diesel exhaust and during exposure to filtered

air) (Table 2). Myocardial ischemia was detected during exercise in all subjects, with greater maximum ST-segment depression during exposure to diesel exhaust than during exposure to filtered air (Table 2 and Fig. 1A and 1B) ( $P<0.05$ ). The ischemic burden induced by exercise was greater during exposure to diesel exhaust (Fig. 1C).

#### VASOMOTOR FUNCTION

There were no significant differences in resting heart rate, blood pressure, or baseline blood flow in the noninfused forearm between or during the two study visits. Although there was a dose-dependent increase in blood flow with each vasodilator ( $P<0.001$  for all comparisons), neither endothelium-dependent nor endothelium-independent vasodilatation was affected by inhalation of diesel exhaust (Fig. 2). Comparison of these data with the findings in a contemporary reference population of healthy male volunteers (mean age,  $53\pm 4$  years) showed impaired vasodilatation in response to acetylcholine ( $P=0.02$ ) but not to sodium nitroprusside (Fig. 2).

#### FIBRINOLYTIC AND INFLAMMATORY MARKERS

There were no significant differences in basal plasma concentrations of t-PA ( $10.5\pm 1.0$  and  $9.5\pm 1.0$  ng per milliliter, respectively) or its endogenous inhibitor, PAI-1 ( $18.8\pm 3.0$  and  $17.0\pm 2.0$  ng per milliliter, respectively), 6 hours after exposure to either diesel exhaust or filtered air. Likewise, leukocyte, neutrophil, and platelet counts and serum C-reactive protein concentrations were not altered at 6 or 24 hours by exposure to diesel exhaust or filtered air. Bradykinin caused a dose-dependent increase in plasma t-PA concentrations (data not shown) and net t-PA release (Fig. 3) in the infused arm ( $P<0.001$  for both comparisons) that was suppressed after exposure to diesel exhaust ( $P=0.009$ ; 35% decrease in the area under the curve).

#### DISCUSSION

We have demonstrated that transient exposure to dilute diesel exhaust, at concentrations occurring in urban road traffic, exacerbates exercise-induced myocardial ischemia and impairs endogenous fibrinolytic capacity in men with coronary heart disease. These findings provide a plausible explanation for the epidemiologic observation that exposure to air pollution is associated with adverse cardiovascular events.

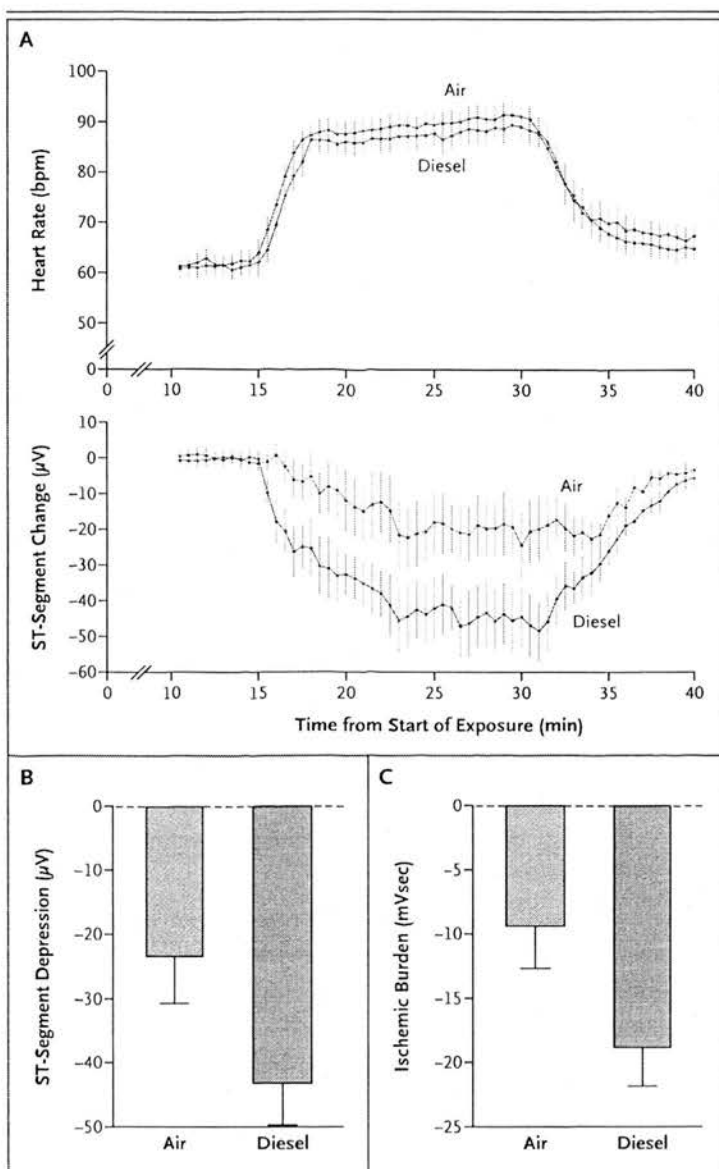


Figure 1. Myocardial Ischemia during 15-Minute Intervals of Exercise-Induced Stress and Exposure to Diesel Exhaust or Filtered Air in the 20 Subjects.

Panel A shows the average change in the heart rate and in the ST segment in lead II. Panel B shows the maximum ST-segment depression during inhalation of diesel exhaust as compared with filtered air ( $P=0.003$ ), and Panel C shows the total ischemic burden during inhalation of diesel exhaust as compared with filtered air ( $P<0.001$ ); the values in Panels B and C are averages of the values in leads II,  $V_2$ , and  $V_5$ . In all three panels, red indicates exposure to diesel exhaust, and blue exposure to filtered air. T bars denote standard errors, and mVsec millivolt seconds.

Concentrations of particulate matter can regularly reach levels of  $300 \mu\text{g}$  per cubic meter in heavy traffic, in occupational settings, and in the world's largest cities.<sup>22</sup> A major proportion of this



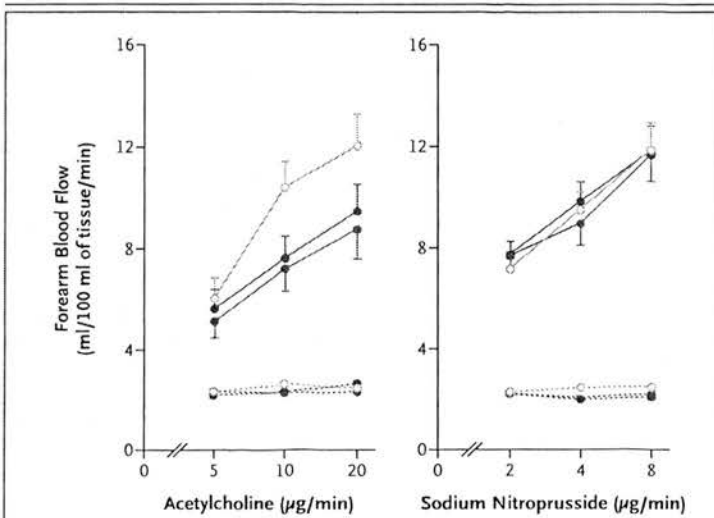


Figure 2. Forearm Blood Flow 6 to 8 Hours after Exposures to Diesel Exhaust and Filtered Air.

Values for infused (solid lines) and noninfused (dashed lines) forearm blood flow are shown for 17 subjects after exposure to diesel exhaust (red) and after exposure to filtered air (blue), as well as for a reference population of matched healthy controls (orange), during intrabrachial infusion of acetylcholine or sodium nitroprusside.  $P < 0.001$  for dose response to both drugs in the infused arm. Among the 17 subjects,  $P = 0.54$  for exposure to diesel exhaust versus filtered air during infusion of acetylcholine, and  $P = 0.56$  during infusion of sodium nitroprusside. For the comparison of the subjects with healthy controls,  $P = 0.02$  during acetylcholine infusion, and  $P = 0.72$  during sodium nitroprusside infusion.

mass is attributable to combustion-derived nanoparticles from traffic, ranging from 20% at remote monitoring sites<sup>23</sup> to 70% in a road tunnel.<sup>24</sup> Exposure to 300 µg of particulate matter per cubic meter for 1 hour increases a person's average exposure over a 24-hour period by only 12 µg per cubic meter. Changes of this magnitude occur on a daily basis, even in the least polluted cities, and are associated with increases in the rate of death from cardiorespiratory disorders.<sup>25</sup> Our model is therefore highly relevant, in terms of both the composition and the magnitude of exposure, to the assessment of short-term health effects in men.

Given potential safety concerns, we recruited patients who had stable and symptomatically well-controlled coronary heart disease, with good exercise tolerance on formal stress testing. The study participants were closely monitored throughout the exposure and reported no adverse effects. Despite similar changes in the heart rate during exposure to diesel exhaust and to filtered air, we documented asymptomatic myocardial ischemia that was increased by a factor of up to three after

inhalation of diesel exhaust. This reproducible effect was present despite extensive use of maintenance beta-blocker therapy in patients without limiting angina. Thus, we have established that inhalation of diesel exhaust has an immediate, proischemic effect, and we believe this provides an important mechanism for the observed increase in myocardial infarction in the hour after exposure to traffic.<sup>13</sup>

Small areas of denudation and thrombus deposition are common findings on the surface of atheromatous plaques and are usually subclinical. Rosenberg and Aird have postulated that vascular-bed-specific defects in hemostasis exist and that propagation of coronary thrombosis is critically dependent on the local fibrinolytic balance.<sup>26</sup> The magnitude and rapidity of t-PA release from the vascular endothelium regulate the generation of plasmin and thus determine the efficacy of endogenous fibrinolysis.

We have previously reported impaired t-PA release in healthy volunteers 6 hours after inhalation of diesel exhaust, although this effect was not seen 2 hours after exposure.<sup>17</sup> We have now confirmed similar reductions in acute t-PA release 6 hours after inhalation of diesel exhaust in patients with coronary heart disease. This delayed effect on endogenous fibrinolysis cannot explain our findings of immediate myocardial ischemia but is consistent with the observations of Peters and colleagues, who reported a second peak in the incidence of myocardial infarction 5 to 6 hours after exposure to traffic.<sup>13</sup> Preclinical thrombotic models also lend support to our findings. Nemmar and colleagues reported that in a hamster model, instillation of diesel-exhaust particulate into the lungs increases venous and arterial thrombus formation at sites of vascular injury.<sup>27</sup> Taken together, these findings indicate an important thrombotic effect of diesel-exhaust inhalation that may promote coronary thrombosis.

Although we found important adverse effects of diesel exhaust on vascular fibrinolytic function, we did not detect an effect on vasomotor function. However, vasomotor function was assessed 6 hours after exposure and 5 hours after we documented an increase in the ischemic burden. We have previously demonstrated that exposure to diesel exhaust impairs vasomotor function in healthy volunteers.<sup>17</sup> This effect was most marked at 2 hours but was still present 6 hours after exposure. Therefore, we cannot exclude the possibil-



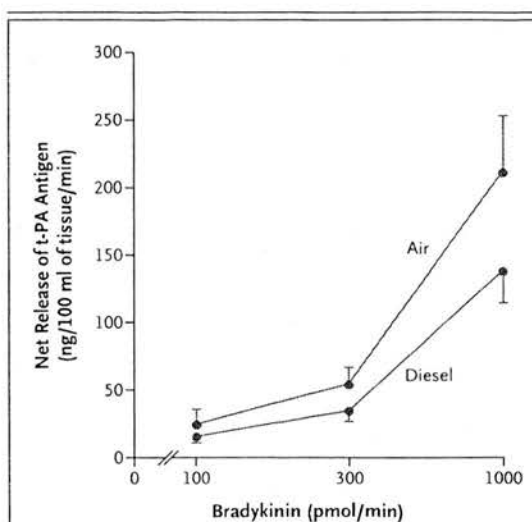
ity of a detrimental vasomotor effect in patients at an earlier point in time.

Patients with coronary heart disease are known to have impaired endothelial function,<sup>28</sup> and we confirm the presence of endothelial dysfunction in our patients. This may have hindered our ability to demonstrate a further impairment of vascular function after exposure to diesel exhaust. In addition, we performed our assessments while the subjects were taking medications that are known to influence endothelial vasomotor function.<sup>29</sup> Furthermore, Brook and colleagues reported that air pollution does not have an effect on endothelium-dependent vasodilatation.<sup>30</sup>

We have identified two distinct and potentially synergistic adverse cardiovascular effects of air pollution in patients with coronary heart disease. These effects may contribute to the increased incidence of myocardial infarction after exposure to traffic. However, the precise mechanisms by which diesel-exhaust inhalation induces these ischemic and thrombotic effects have not been established in our study and will need to be determined in future work.

Our findings are consistent with epidemiologic studies showing associations between ambient particulate air pollution and increased myocardial ischemia during formal exercise testing.<sup>10,11</sup> Myocardial ischemia occurs as a consequence of reduced myocardial oxygen supply, increased demand, or both. We hypothesize that oxidative stress and microvascular dysfunction in the resistance vessels of the myocardium may, in part, explain the adverse ischemic effects of exposure to dilute diesel exhaust. In vitro studies, animal models, and studies of exposures in humans have clearly established the oxidant and proinflammatory nature of combustion-derived particulate matter.<sup>31</sup> Indeed, the pattern of vascular dysfunction in our previous studies suggested that oxidative stress and reduced nitric oxide availability may play a role in mediating the adverse vascular effects of diesel-exhaust inhalation.<sup>17</sup>

Diesel exhaust is a complex mixture of gases and particles, and from our findings, we cannot rule out a nonparticulate cause of the adverse cardiovascular effects. However, on the basis of epidemiologic studies,<sup>32</sup> particulate matter is thought to be responsible for the majority of the adverse health effects of air pollution.<sup>33</sup> This view is supported by the recent observations of Miller and colleagues, who found that cardiovascular out-



**Figure 3. Release of Tissue Plasminogen Activator (t-PA) Antigen 6 to 8 Hours after Exposures to Diesel Exhaust and Filtered Air in 17 Subjects.**

As compared with filtered air (blue), inhalation of diesel exhaust (red) reduced the net release of t-PA antigen (calculated as the product of forearm plasma flow and the difference in t-PA concentrations between the two arms) by 35% ( $P=0.009$ ).

comes were strongly associated with long-term exposure to particulate matter but not with gaseous pollutants.<sup>3</sup> Ambient nitrogen dioxide can be considered a surrogate for pollution from traffic, but it has little adverse effect in controlled-chamber studies, even at the exposure levels in our study.<sup>34</sup> We therefore suggest that the cardiovascular effects described here are mediated primarily by the particulates in diesel exhaust and not by its other components. This argues for the use of diesel-exhaust particle traps to limit the adverse health effects of traffic emissions. However, the causative role of particulates must first be definitively established, and the efficacy of particle traps confirmed.

Brief exposure to dilute diesel exhaust increases myocardial ischemia and impairs endogenous fibrinolytic capacity in men with stable coronary heart disease. Our findings suggest mechanisms for the observation that exposure to combustion-derived air pollution is associated with adverse cardiovascular events, including acute myocardial infarction. Environmental health policy interventions targeting reductions in urban air pollution should be considered in order to decrease the risk of adverse cardiovascular events.

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## **SECTION 4**

### **HEART FAILURE (Publications 26-30)**

**PUBLICATION 26**

# Effects of Acute Angiotensin II Type 1 Receptor Antagonism and Angiotensin Converting Enzyme Inhibition on Plasma Fibrinolytic Parameters in Patients With Heart Failure

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**Background**—Angiotensin converting enzyme (ACE) inhibition after myocardial infarction is associated with an improvement in plasma fibrinolytic parameters. The aim of the present study was to determine whether acute ACE inhibition and angiotensin II type 1 (AT<sub>1</sub>) receptor antagonism have similar effects in patients with heart failure.

**Methods and Results**—Twenty patients with moderately severe chronic heart failure received enalapril 10 mg and losartan 50 mg on 2 separate occasions in a single-blind, randomized, crossover design. Plasma tissue plasminogen activator (t-PA) and plasminogen activator inhibitor type 1 (PAI-1) antigen and activity were measured at baseline and 6 hours after the dose. Acute administration of losartan but not of enalapril reduced plasma t-PA (11%;  $P=0.003$ ) and PAI-1 (38%;  $P<0.001$ ) antigen concentrations, which was associated with increases in t-PA (29%;  $P=0.03$ ) and decreases in PAI-1 (48%;  $P=0.01$ ) activity. Changes in plasma fibrinolytic parameters were more marked during losartan treatment ( $P<0.02$ ), with a 3-fold greater reduction in plasma PAI-1 antigen concentrations ( $P<0.05$ ).

**Conclusions**—Acute AT<sub>1</sub> antagonism in patients with heart failure is associated with a significant improvement in plasma fibrinolytic parameters that is greater than during ACE inhibition. These beneficial effects of AT<sub>1</sub> antagonism and ACE inhibition would therefore appear to be mediated principally through suppression of angiotensin II. (*Circulation*. 1999;99:2983-2985.)

**Key Words:** angiotensin ■ plasminogen activators ■ heart failure ■ fibrinolysis

It would be anticipated that high tissue plasminogen activator (t-PA) concentrations would protect against subsequent coronary events. However, paradoxically, epidemiological studies of total t-PA (antigen) concentrations in patients with ischemic heart disease<sup>1,2</sup> have observed a positive correlation with future coronary events. This may be explained by the concomitant elevation of plasminogen activator inhibitor type 1 (PAI-1), which complexes with t-PA and therefore causes an overall reduction in free t-PA "activity."<sup>3,4</sup> It is this free and unbound t-PA that is physiologically active and leads to endogenous fibrinolysis.

Several large-scale heart failure and post-myocardial infarction trials (VHEFT-II [Veterans Administration Heart Failure Trial II], SAVE [Survival And Ventricular Enlargement], SOLVD [Studies Of Left Ventricular Dysfunction], AIREX [Acute Infarction Ramipril Efficacy eXtension Study], TRACE [TRAndolapril Cardiac Evaluation], and SMILE [Survival of Myocardial Infarction: Long-term Evaluation]) have suggested a reduction in reinfarction rates in patients treated with ACE inhibitors. The mechanisms under-

lying this reduction in coronary thrombotic events are unknown. However, given that angiotensin II<sup>5</sup> and bradykinin<sup>6</sup> are known to induce the release of PAI-1 and t-PA, respectively, the benefits of ACE inhibitor therapy may be mediated through increases in bradykinin-induced t-PA release or a reduction in angiotensin II-mediated PAI-1 release or both. Indeed, the use of ACE inhibitors after myocardial infarction is associated with a decrease in PAI-1 concentrations and a potential increase in t-PA activity.<sup>7,8</sup> However, the effects of ACE inhibition on plasma fibrinolytic factors have not been assessed in patients with heart failure, and it is unknown whether these beneficial effects are also seen with angiotensin II type 1 (AT<sub>1</sub>) receptor antagonism. The aim of the present study, therefore, was to determine whether acute ACE inhibition and AT<sub>1</sub> receptor antagonism have similar effects in patients with heart failure.

## Methods

### Subjects

Twenty patients with New York Heart Association (NYHA) grade II to III chronic heart failure and objective evidence of left ventricular

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TABLE 1. Patient Characteristics

Age, y (mean±SEM)	65±2
Sex, male:female	13:7
Etiology, n	
Ischemic heart disease	18
Dilated cardiomyopathy	2
NYHA grade	
II	6
III	14
Left ventricular ejection fraction, % (mean±SEM)	28±3
Concomitant therapy, n	
Diuretic	15
Aspirin	13
Nitrates	9
β-Blockers	4
Digoxin	3

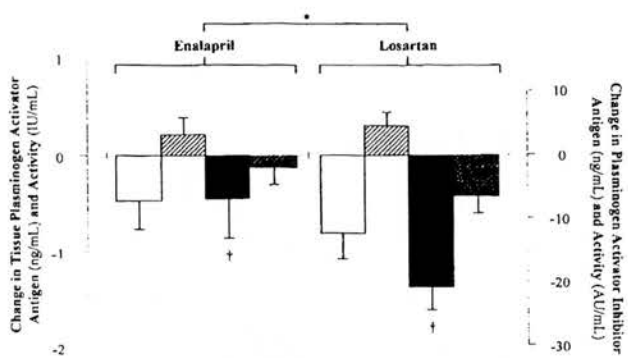
impairment (left ventricular ejection fraction <40%, shortening fraction <20%, or left ventricular end-diastolic diameter >5.5 cm) were recruited with the approval of the local research ethics committee and in accordance with the Declaration of Helsinki. The written informed consent of each subject was obtained before entry into the study. No patient had previously received ACE inhibitor or AT<sub>1</sub> receptor antagonist therapy or had a myocardial infarction within 3 months of the study. Concomitant therapy was omitted on the day of attendance.

Measurements

Supine heart rate and blood pressure were monitored at intervals throughout each study with a semiautomated, noninvasive oscillometric sphygmomanometer<sup>9</sup> (Takeda UA 751, Takeda Medical Inc). Ten milliliters of blood was withdrawn from the antecubital fossa of the forearm and collected into acidified buffered citrate (Biopool Stabilyte for t-PA assays) and citrate (Monovette for PAI-1 assays) tubes and kept on ice before being centrifuged at 2000g for 30 minutes at 4°C. Platelet-free plasma was decanted and stored at -80°C before assay.<sup>10</sup> Plasma PAI-1 and t-PA antigen and activities and atrial natriuretic peptide (ANP) concentrations were determined by ELISAs and a photometric method as previously described.<sup>11,12</sup> All assays were performed by blinded independent operators.

Study Design

Patients attended the clinic at 9 AM and rested recumbent for 20 minutes before measurements were made. At 10 AM, patients



Changes in plasma t-PA (white bars) and PAI-1 (black bars) antigen (solid bars) and activity (hatched bars) after single oral dose of enalapril and losartan in patients with heart failure. \**P*=0.016 (2-way ANOVA for changes in 4 plasma fibrinolytic parameters; losartan vs enalapril); †*P*=0.047 (*t* test for changes in plasma PAI-1 antigen; losartan vs enalapril).

received a single-blind, randomized oral dose of enalapril 10 mg or losartan 50 mg followed by a light lunch at midday. After an additional 20-minute period of supine rest at 4 PM, repeated measurements were taken to coincide with peak plasma concentrations of the active metabolites (enalaprilat and E3174, respectively). To allow for a sufficient washout of enalaprilat and E3174, patients reattended 2 days later to undergo the same protocol but crossed over to receive the alternate therapy.

Data Analysis and Statistics

Data were examined by ANOVA and 2-tailed paired Student's *t* test with Excel version 5.0 (Microsoft). All results are expressed as mean±SEM. Statistical significance was taken at the 5% level.

Results

Patient characteristics are shown in Table 1. Baseline predose hemodynamic, plasma ANP, and fibrinolytic parameters were similar on the 2 study days, with no significant differences (Table 2) or time order effects.

After losartan therapy, plasma t-PA and PAI-1 antigen concentrations fell by 11% (*P*=0.003) and 38% (*P*<0.001), respectively (Table 2; Figure). Plasma t-PA activity increased by 29% (*P*=0.03), whereas PAI-1 activity fell by 48% (*P*=0.01). Enalapril therapy was associated with similar changes in fibrinolytic parameters (-6%, -14%, 21%, and -17%, respectively), but they were not statistically significant (*P*=0.1 to 0.4). Changes in plasma fibrinolytic param-

TABLE 2. Effect of Losartan and Enalapril on Systemic Hemodynamics and Plasma ANP and Fibrinolytic Parameters

	Enalapril			Losartan		
	Predose	Postdose	<i>P</i>	Predose	Postdose	<i>P</i>
Mean arterial pressure, mm Hg	93±4	83±5	*	91±3	88±4	*
Heart rate, bpm	70±3	64±3	*	71±2	66±3	*
t-PA antigen, ng/mL	7.6±0.8	7.1±0.8		7.2±0.5	6.4±0.4	*
t-PA activity, IU/mL	1.0±0.1	1.2±0.2		1.1±0.2	1.4±0.2	†
PAI-1 antigen, ng/mL	47.7±5.9	40.8±4.7		54.6±6.4	33.9±3.8	*
PAI-1 activity, AU/mL	11.3±1.8	9.3±2.0		13.8±2.5	7.2±1.6	†
ANP, ng/mL	17.5±3.1	13.6±1.7		17.4±3.0	11.0±1.1	‡

Values are mean±SEM.  
\**P*≤0.003; †*P*≤0.03; ‡*P*=0.06 (paired *t* test; pre- vs post-dose).

ters were more marked during losartan treatment ( $P=0.016$ ; 2-way ANOVA, enalapril versus losartan), with a 3-fold greater reduction in plasma PAI-1 antigen concentrations ( $P=0.047$ ;  $t$  test, enalapril versus losartan). There were no significant effects on plasma ANP concentrations (Table 2).

### Discussion

We have shown that acute  $AT_1$  receptor antagonism in patients with heart failure is associated with a significant improvement in plasma fibrinolytic parameters. Moreover, this improvement was greater than with ACE inhibition, which suggests that these effects are mediated directly through suppression of angiotensin II.

Previous studies have shown the beneficial effects of ACE inhibition on fibrinolytic parameters in patients after an acute myocardial infarction.<sup>7,8</sup> In the present study, we assessed the acute effects in patients with heart failure, and our results are consistent with these post-myocardial infarction studies. This suggests that the benefits of ACE inhibition are not limited to the immediate postinfarction period but may also be achieved in patients with heart failure. Additionally, we have directly assessed t-PA activity and have confirmed that the changes in basal t-PA and PAI-1 antigen concentrations are associated with an increase in t-PA activity.

It has been suggested that the changes in fibrinolytic parameters seen with ACE inhibitors may be mediated through augmentation of bradykinin, because t-PA release is induced by bradykinin infusions during systemic ACE inhibition.<sup>6</sup> However, the present study would suggest that bradykinin is not involved in basal t-PA release because of the similar effects of  $AT_1$  receptor antagonism on the profile of plasma t-PA and PAI-1 concentrations. Moreover, given the greater efficacy of  $AT_1$  receptor antagonism, particularly on PAI-1 concentrations, it would appear that angiotensin II is the principal mediator of this effect. This is consistent with the *in vitro*<sup>13</sup> and *in vivo*<sup>8</sup> release of PAI-1 with angiotensin II administration. Furthermore, these observations may provide one potential explanation for the findings of the ELITE [Evaluation of Losartan in The Elderly] study, in which  $AT_1$  receptor antagonism was associated with reduced mortality compared with ACE inhibition in patients with heart failure.<sup>14</sup>

Plasma t-PA and PAI-1 concentrations undergo diurnal variations, which have a complementary and inverse sinusoidal relationship.<sup>15</sup> Such diurnal variation may contribute in part to the observed changes in fibrinolytic parameters, although this does not explain the differential effects of ACE inhibition and  $AT_1$  receptor antagonism, especially on PAI-1. In addition, the doses of losartan<sup>14</sup> and enalapril<sup>16</sup> were chosen on the basis of the major published heart failure trials, and the observed differences are unlikely to reflect a dose effect because the resultant hemodynamic effects were similar.

In conclusion, the present study suggests that in patients with heart failure, acute angiotensin II inhibition with  $AT_1$

receptor antagonism produces a marked improvement in basal fibrinolytic balance through a reduction in PAI-1.

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**PUBLICATION 27**

# Potential of Bradykinin-Induced Tissue Plasminogen Activator Release by Angiotensin-Converting Enzyme Inhibition

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<b>OBJECTIVES</b>	The aim of the present study was to determine the effect of angiotensin-converting enzyme (ACE) inhibition on the local stimulated release of tissue plasminogen activator (t-PA) from the endothelium.
<b>BACKGROUND</b>	Angiotensin-converting enzyme inhibitor therapy may exert a beneficial effect on the endogenous fibrinolytic balance.
<b>METHODS</b>	Blood flow and plasma fibrinolytic factors were measured in both forearms of eight healthy males who received unilateral brachial artery infusions of the endothelium-dependent vasodilators substance P (2 to 8 pmol/min) and bradykinin (100 to 1,000 pmol/min), and the endothelium-independent vasodilator sodium nitroprusside (2 to 8 µg/min). These measurements were performed on each of three occasions following one week of matched placebo, quinapril 40 mg or losartan 50 mg daily administered in a double-blind randomized crossover design.
<b>RESULTS</b>	Sodium nitroprusside, substance P and bradykinin produced dose-dependent increases in the blood flow of infused forearm (analysis of variance [ANOVA], $p < 0.001$ for all). Although sodium nitroprusside did not affect plasma t-PA concentrations, they were increased dose-dependently in the infused forearm by substance P and bradykinin infusion (ANOVA, $p < 0.001$ for both). Bradykinin-induced release of active t-PA was more than doubled during treatment with quinapril in comparison to placebo or losartan (two-way ANOVA: $p < 0.003$ for treatment group, $p < 0.001$ for t-PA response and $p = \text{ns}$ for interaction), whereas the substance P response was unaffected.
<b>CONCLUSIONS</b>	We have shown a selective and marked augmentation of bradykinin-induced t-PA release during ACE inhibition. These findings suggest that the beneficial clinical and vascular effects of ACE inhibition may, in part, be mediated through local augmentation of bradykinin-induced t-PA release. (J Am Coll Cardiol 2001;38:1402-8) © 2001 by the American College of Cardiology

The fibrinolytic factor tissue plasminogen activator (t-PA) is a serine protease that regulates the degradation of intravascular fibrin and is released from the endothelium through the translocation of a dynamic intracellular storage pool (1,2). If endogenous fibrinolysis is to be effective then the rapid mobilization of t-PA from the endothelium is essential, because thrombus dissolution is much more effective if t-PA is incorporated during, rather than after, thrombus formation (3). The efficacy of plasminogen activation and fibrin degradation is further determined by the relative balance between the acute local release of t-PA and its subsequent inhibition through formation of complexes with

the serpin plasminogen activator inhibitor type 1 (PAI-1). This dynamic aspect of endothelial function and fibrinolytic balance may be directly relevant to the pathogenesis of atherothrombosis, but only recently have robust methods to determine acute t-PA release been developed (4-7).

Small areas of denudation and thrombus deposition are a common finding on the surface of atheromatous plaques (8,9) and are usually subclinical. However, in the presence of an imbalance in the coagulation or fibrinolytic systems, such microthrombi may propagate, ultimately leading to arterial occlusion (10). Bradykinin is released during the contact phase of coagulation, when high-molecular-weight kininogen is cleaved by kallikrein to produce a disulphide-linked light and heavy chain (11,12). Although an inflammatory mediator, bradykinin is also a potent endothelial cell stimulant that can induce the acute release of t-PA from the endothelium (7,13) through a  $B_2$  receptor mechanism (14). Thus, following activation of the intrinsic coagulation pathway, the liberation of bradykinin may represent an important negative feedback loop in which bradykinin-

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#### Abbreviations and Acronyms

ACE	=	angiotensin-converting enzyme
ANOVA	=	analysis of variance
AT <sub>1</sub>	=	angiotensin II type 1
PAI-1	=	plasminogen activator inhibitor type 1
t-PA	=	tissue plasminogen activator

induced t-PA release inhibits thrombus formation within the vascular lumen when localized endothelial denudation occurs. Furthermore, given that bradykinin-induced forearm vasodilatation is potentiated by angiotensin-converting enzyme (ACE) inhibition (15), such actions may be enhanced in the presence of ACE inhibition and may, in part, explain the anti-ischemic action of this therapy in vascular disease (16). However, although inferred, the potentiation of bradykinin-induced t-PA release by ACE inhibition has yet to be established. Therefore, the aim of the present study was to determine whether systemic ACE inhibition could augment acute local t-PA release from the endothelium *in vivo* in humans.

## METHODS

**Subjects.** Eight healthy male nonsmokers, ages between 21 and 30 years, participated in the study, which was undertaken with the approval of the local research ethics committee, in accordance with the Declaration of Helsinki and with the written informed consent of each subject. Except for study medication, none of the subjects received vasoactive drugs in the week before each phase of the study, and all abstained from alcohol for 24 h and from food and caffeine-containing drinks for at least 4 h before each study. All studies were carried out in a quiet, temperature-controlled room maintained at 22°C to 24°C.

**Measurements. FOREARM BLOOD FLOW AND HEMODYNAMICS.** Blood flow was measured in both forearms by venous occlusion plethysmography using mercury-in-silastic strain gauges applied to the widest part of the forearm as previously described (17). Analogue voltage output from an EC-4 Strain Gauge Plethysmograph (D.E. Hokanson Inc., Bellevue, Washington) was processed by a MacLab analogue-to-digital converter and Chart v3.3.8 software (AD Instruments Ltd., Castle Hill, Australia) and recorded onto a Macintosh Classic II computer (Apple Computers Inc., Cupertino, California). Calibration was achieved using the internal standard of the plethysmograph.

Blood pressure and heart rate were monitored in the noninfused arm at intervals throughout each study using a semiautomated noninvasive oscillometric sphygmomanometer (Takeda UA 751, Takeda Medical Inc., Tokyo, Japan).

**ASSAYS.** Venous cannulae (17G) were inserted into large subcutaneous veins of the antecubital fossa in both arms. Ten to 20 ml of blood was withdrawn simultaneously from each arm and collected into acidified buffered citrate (Biopool Stabilyte, Umeå, Sweden, for t-PA assays), citrate

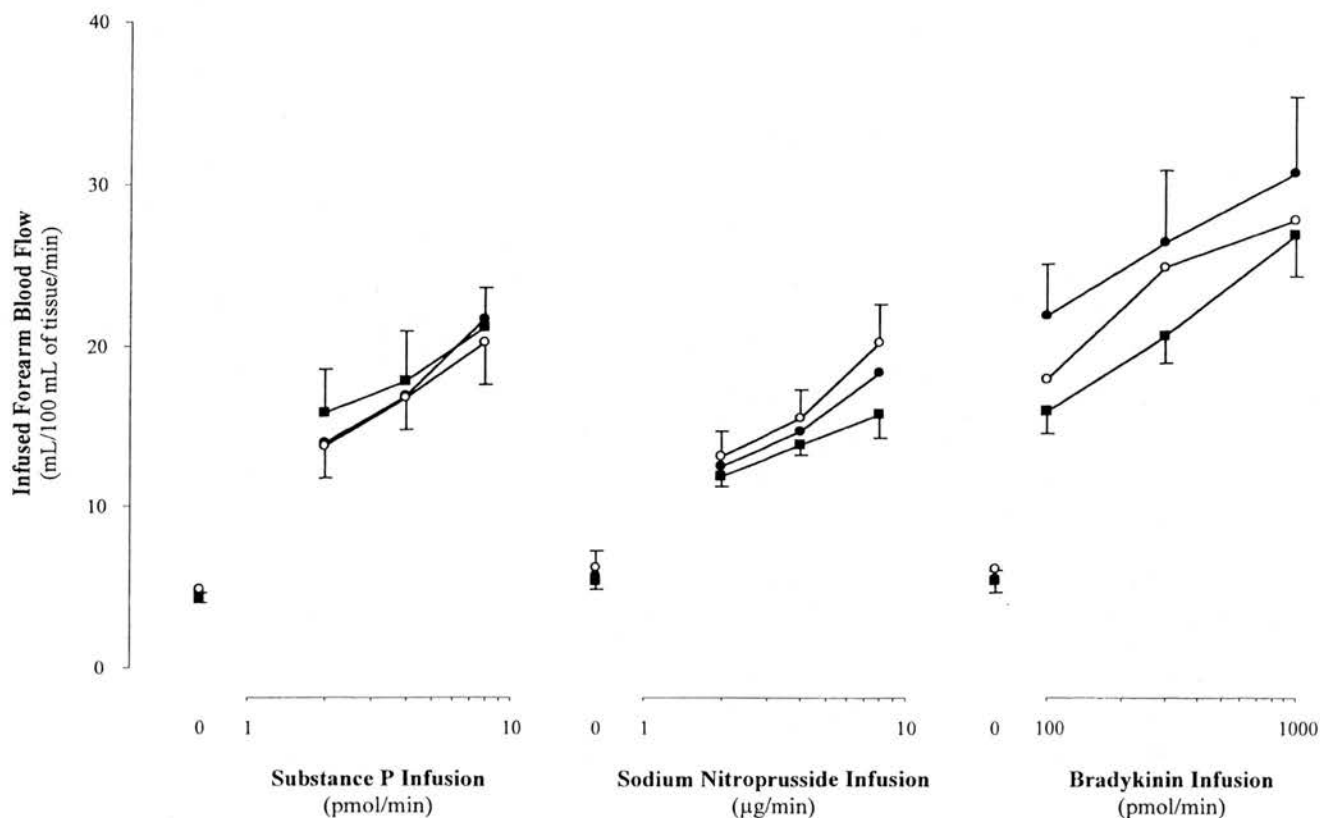
(Monovette, Sarstedt, Nümbrecht, Germany, for PAI-1 assays) and lithium heparin (for ACE activity assays) tubes, and kept on ice before being centrifuged at 2,000 *g* for 30 min at 4°C. Platelet-free plasma was decanted and stored at -80°C before assay (18).

Plasma t-PA and PAI-1 antigen and activity concentrations were determined using enzyme-linked immunosorbent assays and a photometric method as previously described (5,19). Plasma ACE activity was determined using a spectrophotometric technique (Sigma Chemical Corporation, St. Louis, Missouri) (20). Hematocrit was determined by capillary tube centrifugation of blood anticoagulated by ethylene diamine tetraacetic acid.

**Study design.** Subjects attended on each of three study days, two weeks apart, having received matched placebo, quinapril 40 mg and losartan 50 mg (an angiotensin II type 1 (AT<sub>1</sub>) receptor antagonist) once daily for the seven days before attendance. The subjects received each of the medications in a double-blind randomized crossover design. On each study day, the final dose of placebo, quinapril or losartan was taken at 08.00 hr. Six hours later, the subjects rested recumbent, and strain gauges and cuffs were applied to the forearms. The brachial artery of the nondominant arm was cannulated with a 27-standard wire gauge steel needle (Cooper's Needle Works Ltd., Birmingham, United Kingdom) under 1% lidocaine (Xylocaine: Astra Pharmaceutical Ltd., Kings Langley, United Kingdom) local anesthesia. The total rate of intra-arterial infusions was maintained constant at 1 ml/min and forearm blood flow was measured every 10 min throughout all studies. Intrabrachial infusions of substance P (Clinalfa AG, Läufelfingen, Switzerland; endothelium-dependent vasodilator) at 2, 4 and 8 pmol/min (5,19), sodium nitroprusside (David Bull Laboratories, Warwick, United Kingdom; endothelium-independent vasodilator) at 2, 4 and 8 µg/min (5,7) and bradykinin (Clinalfa AG; endothelium-dependent vasodilator) at 100, 300 and 1,000 pmol/min were given for 10 min at each dose in that order. Saline was infused for 30 min before the substance P, sodium nitroprusside and bradykinin infusions.

**Data analysis and statistics.** Plethysmographic data were extracted from the Chart data files. Forearm blood flows were calculated for individual venous occlusion cuff inflations by a template spreadsheet (Excel v5.0; Microsoft Corporation, Cambridge, Massachusetts) as previously described (5,7,19). Estimated net release of t-PA activity and antigen was previously defined (5,7,19) as the product of the infused forearm plasma flow (based on the mean hematocrit and the infused forearm blood flow) and the concentration difference between the infused and noninfused arms. Data were examined, where appropriate, by analysis of variance (ANOVA) with repeated measures and two-tailed paired Student *t* test using Excel v5.0 (Microsoft). All results are expressed as mean ± standard error of the mean. Statistical significance was taken at the 5% level.





**Figure 1.** Infused forearm blood flow response to intrabrachial infusion of substance P, sodium nitroprusside and bradykinin during placebo (open circles), quinapril (closed circles) and losartan (closed squares) administration.  $p < 0.001$  (analysis of variance) for all blood flow responses.

## RESULTS

Oral and intra-arterial drug administrations were well tolerated without significant adverse effects. Consistent with previous studies (7), transient patchy flushing and skin edema of the infused arm occurred with bradykinin infusion at doses  $\geq 300$  pmol/min. There were no significant changes in heart rate, blood pressure and noninfused forearm blood flow (data on file) during or between study days. In comparison to placebo and losartan, plasma ACE activity was suppressed during quinapril administration ( $14.1 \pm 0.8$  IU/ml,  $17.9 \pm 2.4$  IU/ml and  $7.6 \pm 1.6$  IU/ml respectively;  $p < 0.004$ , ANOVA).

**Forearm blood flow responses.** Substance P, sodium nitroprusside and bradykinin produced dose-dependent forearm vasodilatation during each study visit (one-way ANOVA:  $p < 0.001$  for all) (Fig. 1). There were no significant differences between the magnitude of the forearm blood flow responses during placebo, quinapril or losartan administration (two-way ANOVA:  $p = \text{ns}$  for treatment group,  $p < 0.001$  for forearm blood flow response,  $p = \text{ns}$  for interaction). There was a modest trend for bradykinin-induced vasodilatation to be greater during quinapril administration, but this was not statistically significant (two-way ANOVA:  $p = 0.12$  for quinapril vs. placebo,  $p < 0.001$  for forearm blood flow response,  $p = \text{ns}$  for interaction).

**Plasma fibrinolytic parameters.** Substance P and bradykinin caused dose-dependent increases in plasma t-PA antigen and activity concentrations in the infused arm during each study visit (one-way ANOVA:  $p < 0.001$  for all) (Table 1). There was a modest but significant increase in plasma t-PA concentrations in the noninfused arm during infusion of bradykinin, but not substance P. Despite substantial increases in blood flow, sodium nitroprusside had no effect on plasma t-PA concentrations in either arm. During quinapril administration, there was a significant increase in bradykinin, but not substance P, induced increases of plasma t-PA antigen (two-way ANOVA:  $p = 0.05$  for treatment group,  $p < 0.001$  for t-PA response,  $p = \text{ns}$  for interaction) and activity ( $p < 0.001$  for treatment group,  $p < 0.001$  for t-PA response,  $p = \text{ns}$  for interaction) concentrations in the infused forearm (Table 1).

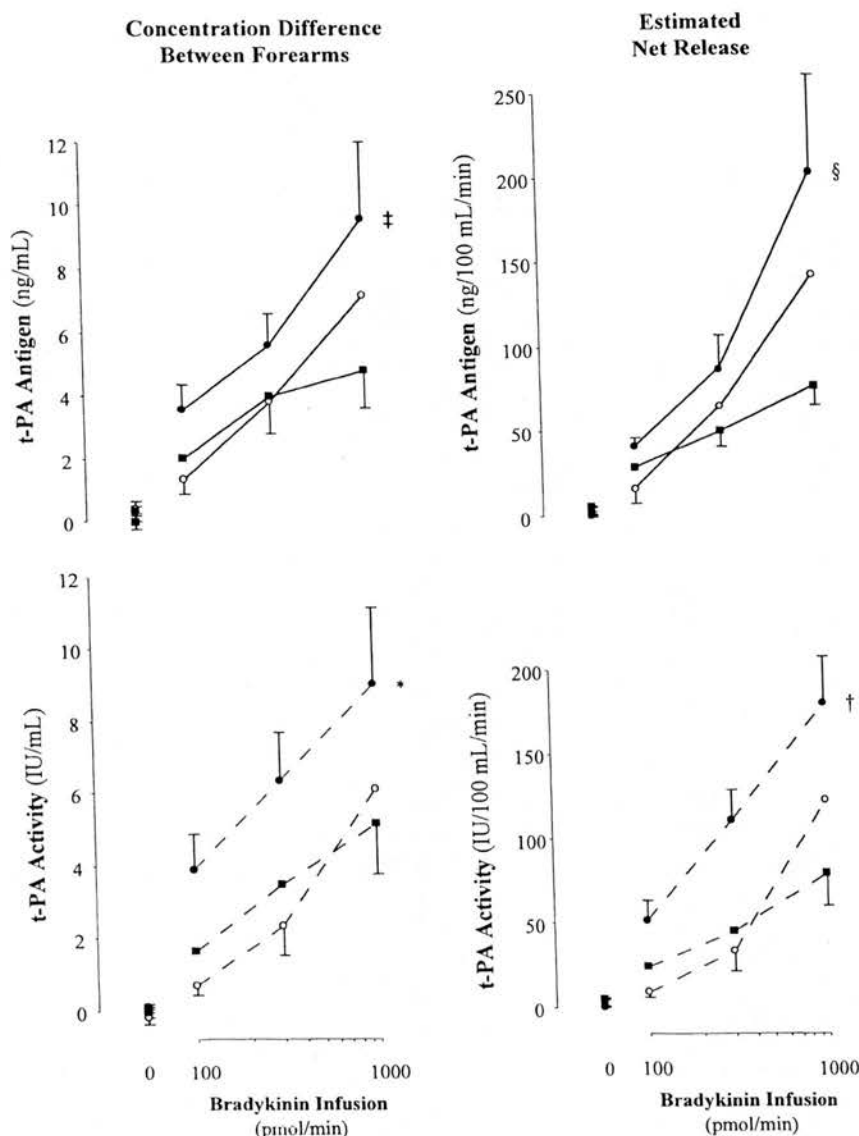
There were no significant differences in basal plasma PAI-1 antigen concentrations during placebo, quinapril or losartan administration, or stimulated release of PAI-1 during substance P, sodium nitroprusside or bradykinin infusion (data on file).

**Release of t-PA.** Substance P and bradykinin caused dose-dependent increases in the plasma t-PA antigen and activity concentration differences between the forearms, and the estimated net release of t-PA antigen and activity during each study visit (one-way ANOVA:  $p < 0.001$  for all).

**Table 1.** Effect of Placebo, Quinapril and Losartan on Plasma t-PA Antigen and Activity Concentrations in the Infused and Noninfused Forearms During Substance P, Sodium Nitroprusside and Bradykinin Infusion

	Substance P (pmol/min)				Sodium Nitroprusside (μg/min)				Bradykinin (pmol/min)			
	0	2	4	8	0	2	4	8	0	100	300	1,000
Placebo												
Plasma t-PA antigen (ng/ml)												
Infused arm	3.9 ± 0.8	4.8 ± 0.8	5.8 ± 0.7	7.5 ± 1.1*	3.3 ± 0.4	3.1 ± 0.5	2.8 ± 0.7	3.8 ± 0.7	3.6 ± 0.6	5.1 ± 0.9	8.0 ± 2.0	13.2 ± 4.4*
Noninfused arm	4.0 ± 0.9	5.3 ± 1.5	4.1 ± 0.8	4.6 ± 1.0	3.9 ± 0.8	3.7 ± 0.8	3.8 ± 0.7	3.4 ± 0.6	3.3 ± 0.5	3.8 ± 1.2	4.2 ± 1.1	6.0 ± 2.3†
Plasma t-PA activity (IU/ml)												
Infused arm	1.8 ± 0.2	2.5 ± 0.4	3.7 ± 0.9	4.8 ± 1.1*	1.9 ± 0.4	1.5 ± 0.1	1.5 ± 0.1	1.6 ± 0.2	1.5 ± 0.2	2.5 ± 0.3	4.5 ± 0.9	8.5 ± 2.0*
Noninfused arm	1.9 ± 0.2	2.0 ± 0.3	1.8 ± 0.2	1.8 ± 0.2	1.8 ± 0.2	1.9 ± 0.3	1.7 ± 0.2	1.7 ± 0.2	1.7 ± 0.2	1.8 ± 0.2	2.2 ± 0.2	2.4 ± 0.4†
Quinapril												
Plasma t-PA antigen (ng/ml)												
Infused arm	2.4 ± 0.3	3.6 ± 0.4	4.2 ± 0.4	6.3 ± 0.7*	3.0 ± 0.3	3.0 ± 0.4	2.6 ± 0.3	2.9 ± 0.5	3.2 ± 0.4	8.0 ± 0.8	11.7 ± 1.7	19.6 ± 6.5\$
Noninfused arm	3.2 ± 0.3	3.3 ± 0.3	3.4 ± 0.4	3.7 ± 0.5	3.0 ± 0.1	2.8 ± 0.3	3.0 ± 0.2	2.9 ± 0.1	2.9 ± 0.1	4.4 ± 0.6	6.2 ± 1.6	10.0 ± 4.3†
Plasma t-PA activity (IU/ml)												
Infused arm	1.3 ± 0.1	2.5 ± 0.4	2.8 ± 0.3	5.2 ± 1.1*	1.6 ± 0.2	1.5 ± 0.1	1.5 ± 0.1	1.5 ± 0.1	1.4 ± 0.1	6.1 ± 1.1	8.9 ± 1.8	12.7 ± 1.9*‡
Noninfused arm	1.4 ± 0.1	1.4 ± 0.1	1.4 ± 0.2	1.5 ± 0.1	1.4 ± 0.2	1.4 ± 0.2	1.3 ± 0.1	1.3 ± 0.1	1.3 ± 0.1	2.2 ± 0.7	2.6 ± 0.8	3.7 ± 1.2†
Losartan												
Plasma t-PA antigen (ng/ml)												
Infused arm	3.4 ± 0.3	4.4 ± 0.7	6.3 ± 1.6	7.6 ± 1.3*	3.7 ± 0.5	3.2 ± 0.2	3.5 ± 0.4	3.7 ± 0.2	3.2 ± 0.3	5.4 ± 0.6	7.9 ± 1.2	9.4 ± 1.4*
Noninfused arm	3.3 ± 0.3	3.6 ± 0.4	3.8 ± 0.4	4.9 ± 0.6	3.6 ± 0.3	3.2 ± 0.3	3.4 ± 0.3	3.2 ± 0.2	3.2 ± 0.3	3.5 ± 0.3	4.0 ± 0.5	4.7 ± 0.6†
Plasma t-PA activity (IU/ml)												
Infused arm	1.4 ± 0.2	3.4 ± 1.1	2.5 ± 0.4	5.2 ± 1.2*	1.6 ± 0.1	1.5 ± 0.1	1.4 ± 0.1	1.4 ± 0.1	1.5 ± 0.1	3.3 ± 0.4	5.3 ± 0.7	7.4 ± 1.4*
Noninfused arm	1.6 ± 0.2	1.7 ± 0.1	1.8 ± 0.2	2.7 ± 0.5	1.5 ± 0.1	1.6 ± 0.1	1.4 ± 0.1	1.4 ± 0.1	1.6 ± 0.2	1.7 ± 0.1	1.8 ± 0.1	2.2 ± 0.3†

\*p < 0.001; †p < 0.05; ‡p < 0.001; §p = 0.05. One-way analysis of variance dose response; Two-way analysis of variance: quinapril versus losartan and placebo (p < 0.001 for t-PA response and p = ns for interaction).  
t-PA = tissue plasminogen activator.



**Figure 2.** Forearm concentration difference (left) and estimated net release (right) of tissue plasminogen activator (t-PA) antigen (upper, solid lines) and activity (lower, dashed lines) in response to intrabrachial infusion of bradykinin during placebo (open circles), quinapril (closed squares) and losartan (closed circles) administration.  $p < 0.001$  (analysis of variance, ANOVA) for all responses. \* $p = 0.003$ , † $p = 0.03$ , ‡ $p = 0.09$ , § $p = 0.13$  two-way ANOVA: quinapril versus losartan and placebo ( $p < 0.001$  for t-PA response and  $p = ns$  for interaction).

(Fig. 2). During quinapril administration, there was a significant increase in bradykinin- but not substance P-induced release of active t-PA (two-way ANOVA:  $p = 0.003$  for treatment group,  $p < 0.001$  for t-PA response,  $p = ns$  for interaction) (Fig. 2) and a trend for t-PA antigen (two-way ANOVA:  $p = 0.09$  for treatment group,  $p < 0.001$  for t-PA response,  $p = ns$  for interaction) (Fig. 2). Quinapril increased the area under the curve for the net release of active t-PA by 135% and 125% in comparison to placebo and losartan respectively.

## DISCUSSION

For the first time, we have shown that in contrast to  $AT_1$  receptor antagonism, ACE inhibition potentiates bradykinin-

induced endogenous t-PA release from the endothelium. However, this potentiation appears to be specific to bradykinin because ACE inhibition did not appear to influence substance-P-induced t-PA release. These findings suggest that the beneficial clinical and vascular effects of ACE inhibition (16) may, in part, be mediated through the acute local augmentation of bradykinin-induced t-PA release.

**Endogenous fibrinolysis and acute coronary syndromes.** The endogenous fibrinolytic system can have important clinical effects, as exemplified by the observation that in a third of patients with an acute myocardial infarction, the infarct-related artery spontaneously reperfuses within 12 h (21-23). Moreover, a low fibrinolytic activity is associated with an increased risk of myocardial infarction in young

men (24) and predicts which patients with unstable angina will develop myocardial infarction (25). Clinical studies of patients with unstable angina have also indicated that there is enhanced activation of the kallikrein system and that bradykinin release is increased (26). Given this augmentation of bradykinin generation and the activation of the intrinsic coagulation pathway in acute coronary syndromes, ACE inhibition may have major beneficial effects on the acute local fibrinolytic balance by markedly enhancing bradykinin-induced t-PA release in areas of intravascular thrombus formation.

**ACE inhibition, bradykinin metabolism and t-PA release.** More than 95% of bradykinin metabolism occurs through ACE, whereas plasma substance P is metabolized by several additional enzymes including dipeptidyl(amino)-peptidase IV and aminopeptidase M (27). Moreover, the tissue and cellular metabolism of substance P is performed almost exclusively by neutral endopeptidase 24.11 (27). Consistent with this, and with previous work in the forearm circulation (28), we did not detect a significant influence of ACE inhibition on substance-P-induced vasodilatation. Indeed, ACE inhibition did not appear to influence substance-P-induced t-PA release. This indicates that the augmentation of bradykinin-induced t-PA release reflects the inhibition of bradykinin metabolism rather than a general enhancement of the capacity of the endothelium to release t-PA acutely. Given the recent report (29) of even greater reductions in cardiovascular events with omapatrilat, a combined ACE and neutral endopeptidase inhibitor, the potentiation of bradykinin-induced t-PA release may be enhanced even further by such compounds. This requires further investigation.

In contrast to the marked potentiation of t-PA release, we failed to detect a significant increase in bradykinin-induced vasodilatation during ACE inhibition. Benjamin et al. (15) have previously reported that local ACE inhibition causes a potentiation of the increases in blood flow associated with bradykinin infusion in the human forearm. However, there did appear to be a trend toward an enhanced blood flow response, and it is likely that our study lacked sufficient power to detect this difference. This would also suggest that, in comparison to vasomotor responses, ACE inhibition has a proportionately greater effect on the enhancement of bradykinin-induced t-PA release given more than a doubling of the release of active t-PA. In addition, it is unlikely that the augmentation of active t-PA release by ACE inhibition is due to the potentially greater increase in blood flow because vasodilatation and increased blood flow do not appear, by themselves, to cause t-PA release, as demonstrated by the absence of an effect with sodium nitroprusside infusion. This latter observation is in agreement with our previous studies (5) and work by other groups (4,6).

**Conclusions.** We have demonstrated a specific augmentation of bradykinin-induced t-PA release by ACE inhibition. This effect appears to be independent of angiotensin II

action because AT<sub>1</sub> receptor antagonism did not influence the acute release of t-PA. These findings suggest that the beneficial clinical and vascular effects of ACE inhibition may, in part, be mediated through the acute local augmentation of bradykinin-induced t-PA release.

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**PUBLICATION 28**

## Angiotensin Antagonists and Heart Failure

# Marked Bradykinin-Induced Tissue Plasminogen Activator Release in Patients With Heart Failure Maintained on Long-Term Angiotensin-Converting Enzyme Inhibitor Therapy

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<b>OBJECTIVES</b>	The aim of the present study was to assess the contribution of angiotensin-converting enzyme (ACE) inhibitor therapy to bradykinin-induced tissue-type plasminogen activator (t-PA) release in patients with heart failure (HF) secondary to ischemic heart disease.
<b>BACKGROUND</b>	Bradykinin is a potent endothelial cell stimulant that causes vasodilatation and t-PA release. In large-scale clinical trials, ACE inhibitor therapy prevents ischemic events.
<b>METHODS</b>	Nine patients with symptomatic HF were evaluated on two occasions: during and following seven-day withdrawal of long-term ACE inhibitor therapy. Forearm blood flow was measured using bilateral venous occlusion plethysmography. Intrabrachial bradykinin (30 to 300 pmol/min), substance P (2 to 8 pmol/min), and sodium nitroprusside (1 to 4 pmol/min) were infused, and venous blood samples were withdrawn from both forearms for estimation of fibrinolytic variables.
<b>RESULTS</b>	On both study days, bradykinin and substance P caused dose-dependent vasodilatation and release of t-PA from the infused forearm ( $p < 0.05$ by analysis of variance [ANOVA]). Long-term ACE inhibitor therapy caused an increase in forearm vasodilatation ( $p < 0.05$ by ANOVA) and t-PA release ( $p < 0.001$ by ANOVA) during bradykinin, but not substance P, infusion. Maximal local plasma t-PA activity concentrations approached 100 IU/ml, and maximal forearm protein release was $\sim 4.5 \mu\text{g}/\text{min}$ .
<b>CONCLUSIONS</b>	Long-term ACE inhibitor therapy augments bradykinin-induced peripheral vasodilatation and local t-PA release in patients with HF due to ischemic heart disease. Local plasma t-PA activity concentrations approached those seen during systemic thrombolytic therapy for acute myocardial infarction. This may contribute to the primary mechanism of the anti-ischemic effects associated with long-term ACE inhibitor therapy. (J Am Coll Cardiol 2002;40: 961-6) © 2002 by the American College of Cardiology Foundation

Bradykinin is a potent endothelium-dependent vasodilator that has a brief duration of action due to its rapid degradation by angiotensin-converting enzyme (ACE). In addition to acting as an inflammatory mediator, bradykinin is closely involved in fibrinolytic and coagulation cascades. During the

feedback loop in which bradykinin-induced t-PA release inhibits intravascular thrombus formation.

Large-scale clinical trials of patients with heart failure (HF) or ischemic heart disease indicate a reduction in recurrent infarction rates with ACE inhibitor therapy (3). The mechanisms underlying this anti-ischemic benefit may relate, in part, to the effects on endogenous fibrinolysis. Inhibition of ACE enhances bradykinin-induced vasodilatation and endothelial t-PA release in healthy volunteers (2). However, to date, there has been no assessment of the effect of long-term ACE inhibition on acute endogenous t-PA release in patients with HF or ischemic heart disease. Therefore, the aim of this study was to determine whether long-term ACE inhibition potentiates acute t-PA release in patients with HF secondary to ischemic heart disease.

See page 967

contact phase of blood coagulation, it is released after cleavage of high-molecular-weight kininogen by kallikrein (1). It is also a potent stimulant for the release of tissue-type plasminogen activator (t-PA) from the endothelium (2). Thus, when plaque rupture or erosion activates the intrinsic coagulation pathway, liberation of bradykinin may represent an important negative

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## METHODS

**Patients.** Nine patients with New York Heart Association functional class II or III HF secondary to ischemic heart

# Abbreviations and Acronyms

ACE	= angiotensin-converting enzyme
ANOVA	= analysis of variance
d.f.	= degree of freedom
HF	= heart failure
MI	= myocardial infarction
PAI-1	= plasminogen activator inhibitor type 1
t-PA	= tissue-type plasminogen activator

disease participated in the study, which was undertaken with the approval of the local Research Ethics Committee, in accordance with the Declaration of Helsinki, and each subject gave written, informed consent. All subjects had been maintained on a maximally tolerated dose of an ACE inhibitor for more than six months, and they abstained from alcohol for 24 h and from food and caffeine-containing drinks for at least 4 h before each study.

**Measurements. FOREARM BLOOD FLOW AND HEMODYNAMICS.** Blood flow was measured in both forearms by venous occlusion plethysmography using mercury-in-Silastic strain gauges applied to the widest part of the forearm, as previously described (2,4). Blood pressure and heart rate were monitored in the noninfused arm at intervals throughout each study by using a semi-automated noninvasive oscillometric sphygmomanometer (Takeda UA 751, Takeda Medical Inc., Tokyo, Japan).

**ASSAYS.** Venous cannulae (17-gauge) were inserted into large subcutaneous veins of the antecubital fossa in both arms. Ten to 20 ml of blood was withdrawn simultaneously from each arm and collected into acidified buffered citrate (Biopool Stabilyte, Umeå, Sweden; for t-PA assays) and citrate (Monovette, Sarstedt, Nümbrecht, Germany; for plasminogen activator inhibitor type 1 [PAI-1] assays) tubes and kept on ice before being centrifuged at 2,000 *g* for 30 min at 4°C. Platelet-free plasma was decanted and stored at –80°C before assay. Plasma t-PA and PAI-1 antigen and activity concentrations were determined using enzyme-linked immunosorbent assays and a photometric method, as previously described (2,4).

**Study design.** Patients were evaluated at 9 AM on two occasions: during and after seven-day withdrawal of long-term ACE inhibitor therapy. On the appropriate study day, oral ACE inhibitor therapy was administered at 8 AM. The brachial artery of the nondominant arm was cannulated with a standard 27-gauge steel-wire needle (Cooper's Needle Works Ltd., Birmingham, UK) under local anesthesia. The total rate of intra-arterial infusions was maintained constant at 1 ml/min, and forearm blood flow was measured every 10 min throughout all studies. Intrabrachial infusions of substance P (Clinalfa AG, Läufelfingen, Switzerland) at 2, 4, and 8 pmol/min, bradykinin (Clinalfa AG) at 30, 100, and 300 pmol/min, and sodium nitroprusside (David Bull Laboratories, Warwick, UK) were given at 1, 2, and 4 µg/min for 10 min at each dose in a randomized order (2). Saline

**Table 1.** Patient characteristics (n = 9)

Age yrs (range)	65 (53–79)
Gender (male/female)	9/0
Concomitant medications (n)	
ACE inhibition	9
Aspirin	8
Diuretic	6
Beta-blocker	2
Statin	8
Nitrate	4
Digoxin	1
Calcium antagonist	1
Spironolactone	1
Warfarin	3
Echocardiographic parameters	
Ejection fraction (%)	25 ± 5
Shortening fraction (%)	10 ± 2
LVEDD (mm)	64 ± 2
Heart rate (beats/min)	
Visit 1	61 ± 5
Visit 2	61 ± 4
Mean arterial pressure* (mm Hg)	
Visit 1	84 ± 4
Visit 2	89 ± 5
Forearm blood flow (ml/100 ml/min)	
Visit 1	
Noninfused	2.3 ± 0.2
Infused	2.3 ± 0.2
Visit 2	
Noninfused	2.8 ± 0.6
Infused	2.6 ± 0.3

Data are presented as the number of patients or mean value ± SEM. \*Diastolic pressure + one-third of pulse pressure.

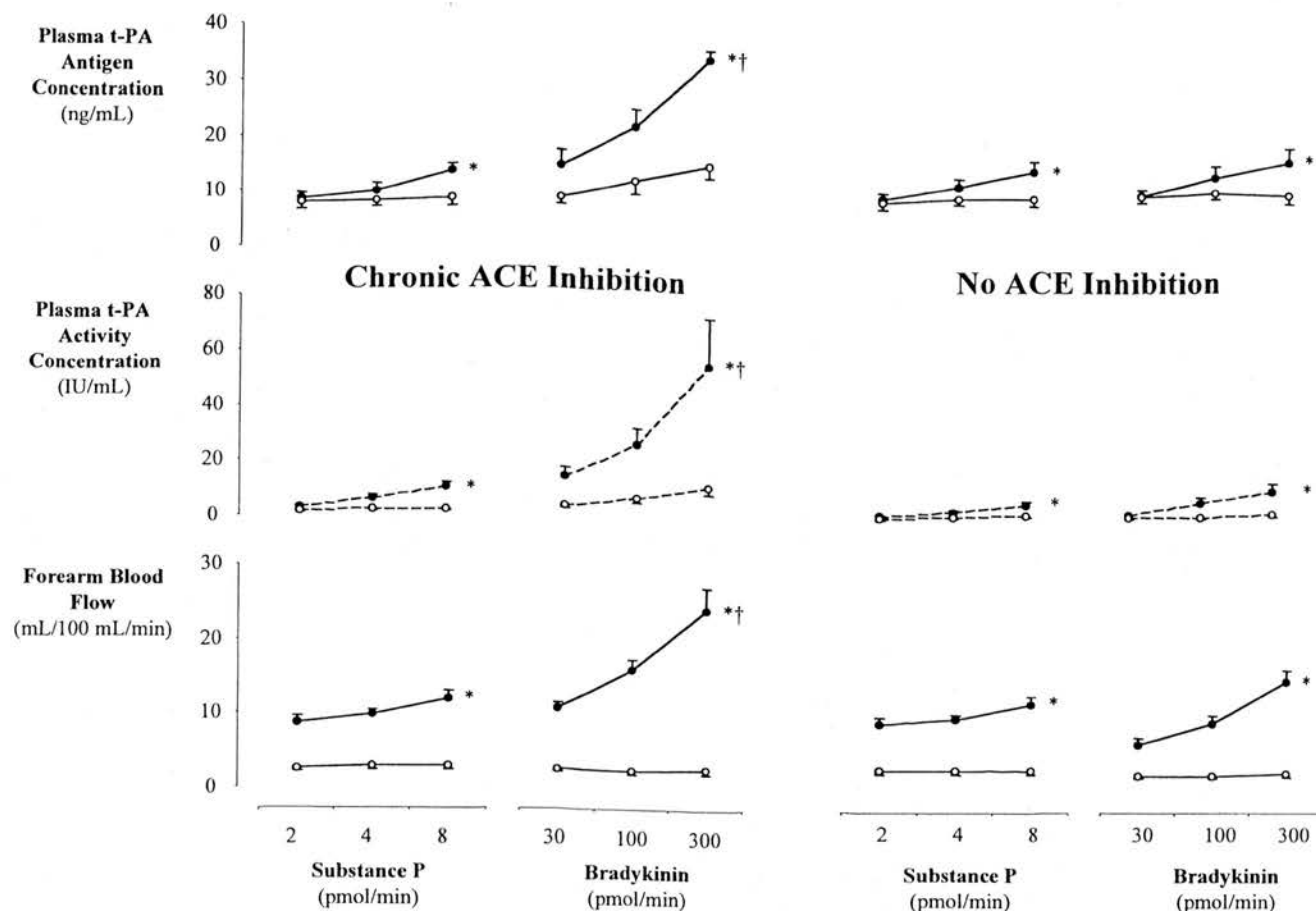
ACE = angiotensin-converting enzyme; LVEDD = left ventricular end-diastolic diameter; Visit 1 = during long-term ACE inhibition; Visit 2 = after seven-day withdrawal of long-term ACE inhibition.

was infused for 30 min before the substance P, sodium nitroprusside, and bradykinin infusions.

**Data analysis and statistics.** Forearm blood flow was calculated for individual venous occlusion cuff inflations, as previously described (2,4). Estimated net release of t-PA activity and antigen was previously defined as the product of the infused forearm plasma flow (based on the mean hematocrit and infused forearm blood flow) and the concentration difference between the infused and noninfused arms (2,4). Data were examined, where appropriate, by analysis of variance (ANOVA) with repeated measures and the two-tailed paired Student *t* test, using Microsoft Excel 97. All results are expressed as the mean value ± SEM. Statistical significance was set at the 5% level.

## RESULTS

Patient characteristics are shown in Table 1. After withdrawal of ACE inhibitor therapy, baseline mean arterial pressure appeared to rise, but this was not statistically significant. There were no significant changes in heart rate, blood pressure or noninfused forearm blood flow (Table 1, Fig. 1) (data on file, University of Edinburgh) during or between the study days.



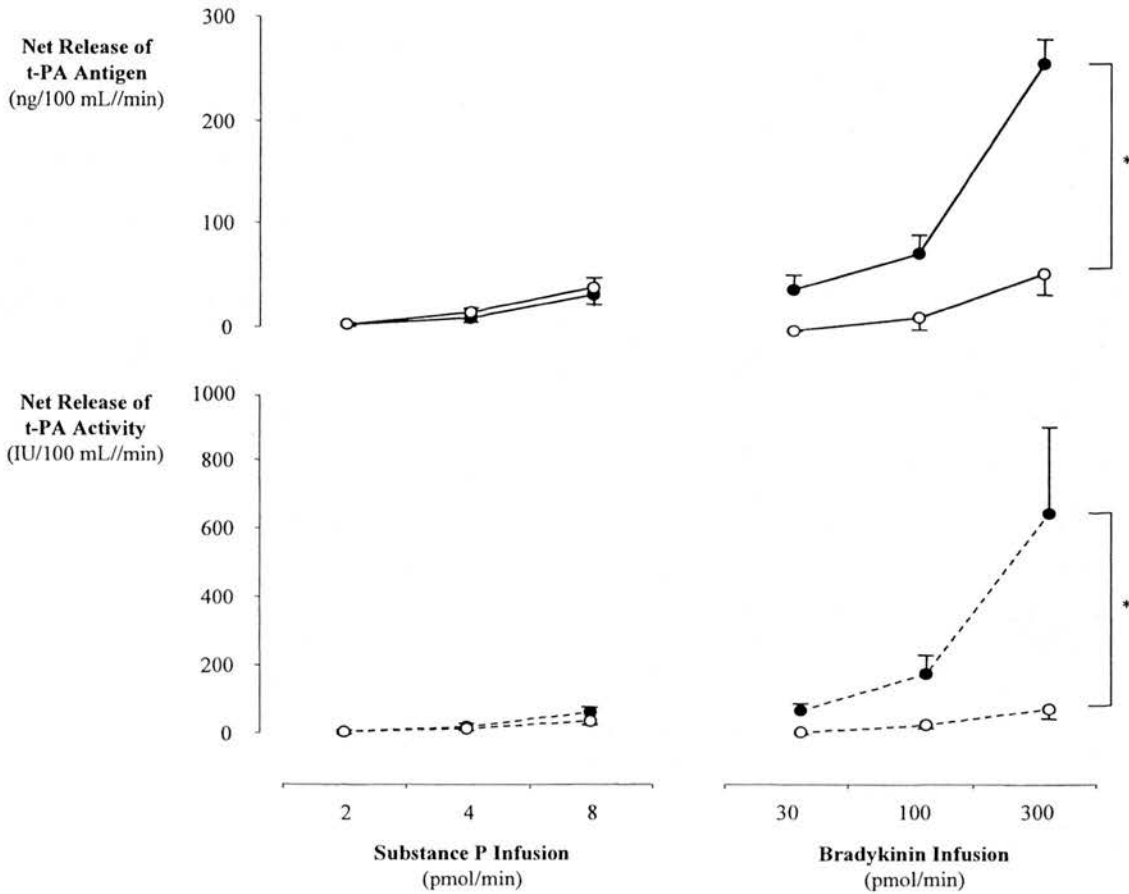
**Figure 1.** Effect of intra-arterial bradykinin and substance P infusions on blood flow and plasma tissue-type plasminogen activator (t-PA) antigen (solid lines) and activity (dashed lines) concentrations in the infused (solid symbols) and noninfused (open symbols) arms in the presence (left) or absence (right) of angiotensin-converting enzyme (ACE) inhibition. \* $p < 0.05$  by analysis of variance (ANOVA). † $p < 0.001$  by ANOVA for long-term ACE inhibition versus no ACE inhibition.

**Forearm blood flow responses.** Sodium nitroprusside (data on file), substance P, and bradykinin caused dose-dependent forearm vasodilatation during each study visit (ANOVA for blood flow response,  $p < 0.001$  for all;  $n = 9$  at baseline and with the three doses, degree of freedom (d.f.) = 3) (Fig. 1). In the presence of long-term ACE inhibition, forearm vasodilatation was augmented during bradykinin (ANOVA for ACE inhibition vs. no ACE inhibition,  $p < 0.05$ ;  $n = 9$  for blood flow at baseline and with the three doses, d.f. = 1), but not substance P or sodium nitroprusside ( $p = \text{NS}$ ) infusion.

**Plasma fibrinolytic variables.** Bradykinin and substance P caused dose-dependent increases in plasma t-PA antigen and activity concentrations in the infused arm (ANOVA for plasma t-PA concentrations,  $p < 0.05$  for all;  $n = 9$  at baseline and with the three doses, d.f. = 3) (Fig. 1). Plasma t-PA antigen and activity concentrations were significantly augmented during bradykinin (ANOVA for ACE inhibition vs. no ACE inhibition,  $p < 0.001$ ;  $n = 9$  for plasma t-PA concentrations at baseline and with the three doses, d.f. = 1), but not substance P ( $p = \text{NS}$ ) infusion in the presence of long-term ACE inhibition.

Basal plasma PAI-1 antigen concentrations were lower in the presence ( $38 \pm 6$  ng/mL) than in the absence ( $48 \pm 7$  ng/mL) of ACE inhibitor therapy (paired  $t$  test for ACE inhibition vs. no ACE inhibition,  $p < 0.02$ ;  $n = 9$  for plasma PAI-1 concentrations, d.f. = 1). Bradykinin and substance P administration had no effect on plasma PAI-1 concentrations (data on file, University of Edinburgh).

**Release of t-PA.** Substance P and bradykinin caused dose-dependent increases in the plasma t-PA antigen and activity concentration differences between the forearms, as well as the estimated net release of t-PA antigen and activity during each study visit (ANOVA for t-PA release,  $p < 0.01$  for all;  $n = 9$  for t-PA release or concentration difference at baseline and with the three doses, d.f. = 3) (Fig. 2). During ACE inhibition, there was a massive increase in bradykinin-induced (ANOVA for ACE inhibition vs. no ACE inhibition,  $p < 0.001$ ;  $n = 9$  for t-PA release at baseline and with the three doses, d.f. = 1) (Fig. 2), but not substance P-induced ( $p = \text{NS}$ ), release of t-PA antigen and activity (increases in the area under the curve of 520% and 877%, respectively). Post-hoc analysis identified no significant effect of other concomitant medica-



**Figure 2.** Estimated net release of tissue-type plasminogen activator (t-PA) antigen (solid lines) and activity (dashed lines) during bradykinin (right) and substance P (left) infusions in the presence (solid circles) or absence (open circles) of angiotensin-converting enzyme (ACE) inhibition.  $p < 0.01$  by analysis of variance (ANOVA) for all responses.  $p < 0.001$  by ANOVA for long-term ACE inhibition versus no ACE inhibition.

tions on substance P-induced or bradykinin-induced t-PA release.

## DISCUSSION

For the first time, to the best of our knowledge, we have shown that in patients with HF secondary to ischemic heart disease, long-term ACE inhibition markedly potentiates bradykinin-induced endogenous t-PA release from the endothelium. However, this potentiation appears to be specific to bradykinin, because ACE inhibition did not influence substance P-induced t-PA release. These findings suggest that the beneficial clinical and vascular effects of ACE inhibition may be partly mediated through the acute local augmentation of bradykinin-induced t-PA release.

**Magnitude of t-PA release.** Long-term ACE inhibition produced a massive augmentation of bradykinin-induced t-PA release across the forearm vascular bed. The approximate fivefold increase in t-PA antigen release and the ~20% reduction in plasma PAI-1 antigen concentrations led to the approximate ninefold increase in the release of active t-PA. Our group has previously shown that bradykinin-induced t-PA release is augmented by ACE

inhibition in healthy volunteers, but this was modest at approximately twofold only (2). The dramatic potentiation of active t-PA release in the present study is exemplified by the observation that the maximal local forearm concentrations of active t-PA (99 IU/ml at 300 pmol/min of bradykinin) approached those observed during systemic thrombolysis during acute myocardial infarction (MI) (100 to 1,000 IU/ml) (5). Moreover, it also underscores the large capacity of the endothelium to release t-PA quickly—up to 4.5  $\mu$ g or 16,000 IU/min from the infused forearm at 300 pmol/min of bradykinin. Indeed, using intrabrachial substance P infusions, we have previously demonstrated substantial and sustained release of t-PA for up to 2 h (6).

Minai et al. (7) have recently reported that ACE inhibition produces an approximate twofold increase in bradykinin-induced t-PA release in the coronary circulation of patients with atypical chest pain and angiographically normal coronary arteries. This is consistent with our previous findings in the peripheral circulation of healthy volunteers (2) and suggests that comparable endothelial fibrinolytic effects exist between the peripheral and coronary circulations (8). The present study extends these previous findings, because we have demonstrated a more marked



augmentation of peripheral t-PA release in patients with HF secondary to ischemic heart disease.

**Mechanism of bradykinin-induced t-PA release.** In keeping with our previous work (2) and that by others (9), ACE inhibition augmented the vasodilatation induced by bradykinin but did not affect the vasodilatation or t-PA release produced by substance P. This suggests that the effect of ACE inhibition does not appear to reflect a generalized enhancement of vascular function but is specific to bradykinin. Brown et al. (10) and Gainer et al. (11) have previously investigated the mechanism of bradykinin-induced t-PA release in the human forearm. Bradykinin induces t-PA release through a B<sub>2</sub> receptor-dependent, nitric oxide synthase-independent, and cyclooxygenase-independent pathway (10). Brown et al. (10) have suggested that bradykinin-induced t-PA release may be caused by an endothelium-derived hyperpolarizing factor, although other mediators may be involved. This group has also described a potential interaction between the vascular responses to bradykinin and the ACE gene insertion/deletion polymorphism (11). We have not explored this interaction because of the small sample size of our study, but this may markedly influence the fibrinolytic response to long-term ACE inhibition in patients with HF or vascular disease, and it requires further investigation.

Inflammation plays an important role in the pathogenesis of HF (12), with elevated plasma concentrations of circulating cytokines such as tumor necrosis factor- $\alpha$  (13). Bradykinin receptor expression is altered by ACE inhibition (14,15), inflammation (16), and chronic HF (14,15,17) and may partly explain the proportionately greater and massive release of t-PA from the endothelium.

**Endothelial function, endogenous fibrinolysis, and HF.** The endothelium plays a vital role in the control of blood flow, hemostasis, fibrinolysis, and inflammation. Consequently, the maintenance and regulation of tissue perfusion critically depends on the integrity of endothelial function and the release of potent endothelium-derived factors. After the seminal work of Furchgott and Zawadzki (18), it has been widely recognized that an array of mediators can influence vascular tone through endothelium-dependent actions, and there is now extensive evidence of abnormal endothelium-dependent vasomotion that is reversed by ACE inhibition in patients with HF (19,20). However, although endothelium-dependent vasomotion is important, it may not be representative of other aspects of endothelial function, such as the regulation of endogenous fibrinolysis.

Tissue plasminogen activator is a serine protease that regulates the degradation of intravascular fibrin and is released from the endothelium through the translocation of a dynamic intracellular storage pool. If endogenous fibrinolysis is to be effective, then the rapid mobilization of t-PA from the endothelium is essential, because thrombus dissolution is much more effective if t-PA is incorporated during, rather than after, thrombus formation (21). This dynamic

aspect of endothelial function and fibrinolytic balance may be directly relevant to the pathogenesis of atherothrombosis and is not necessarily reflected by the basal plasma concentrations of t-PA (22-24).

**Clinical relevance.** The endogenous fibrinolytic system can have important clinical effects, as exemplified by the observation that in one-third of patients with an acute MI, the infarct-related artery spontaneously reperfuses within 12 h (25-27). Moreover, low fibrinolytic activity is associated with an increased risk of MI in young men (28) and predicts which patients with unstable angina will develop MI (29). Clinical studies of patients with unstable angina have also indicated that there is an enhanced activation of the kallikrein system and that bradykinin release is increased (30). Given this augmentation of bradykinin generation and activation of the intrinsic coagulation pathway in acute coronary syndromes, ACE inhibition may have major beneficial effects on the acute local fibrinolytic balance by markedly enhancing bradykinin-induced t-PA release in areas of intravascular thrombus formation. This is consistent with the observation that ACE inhibition improves the basal fibrinolytic balance (31,32) and reduces myocardial troponin release in patients with acute coronary syndromes (33).

**Conclusions.** Long-term ACE inhibitor therapy augments bradykinin-induced peripheral vasodilatation and local t-PA release in patients with HF due to ischemic heart disease. Local plasma t-PA activity concentrations approached those seen during systemic thrombolytic therapy for acute MI. This may contribute to the primary mechanism of the anti-ischemic effects associated with long-term ACE inhibitor therapy.

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**PUBLICATION 29**

# Neutral Endopeptidase Inhibition Augments Vascular Actions of Bradykinin in Patients Treated With Angiotensin-Converting Enzyme Inhibition

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**Abstract**—Angiotensin-converting enzyme and neutral endopeptidase (EC 3.4.24.11; neprilysin) are metallopeptidases present on the endothelium that metabolize bradykinin. Inhibitors of angiotensin-converting enzyme potentiate bradykinin-mediated vasodilatation and endothelial tissue plasminogen activator release. Combined angiotensin-converting enzyme and neutral endopeptidase inhibition may have additional beneficial cardiovascular effects mediated through bradykinin potentiation. We investigated the effects of local neutral endopeptidase inhibition on the vascular actions of bradykinin in heart failure patients maintained on chronic angiotensin-converting enzyme inhibition. Ten patients received intrabrachial infusion of thiorphan (30 nmol/min), a neutral endopeptidase inhibitor, in a randomized double-blind placebo-controlled crossover trial. Thiorphan was coin infused with Lys-des-Arg<sup>9</sup>-bradykinin (1 to 10 nmol/min), bradykinin (30 to 300 pmol/min), atrial natriuretic peptide (10 to 100 pmol/min), and sodium nitroprusside (2 to 8  $\mu$ g/min). Bradykinin, atrial natriuretic peptide, and sodium nitroprusside caused dose dependent vasodilatation (peak blood flow  $14.4 \pm 2.2$ ,  $3.6 \pm 0.6$ , and  $8.6 \pm 1.3$  mL per 100 mL/min, respectively;  $P < 0.0001$ ). Bradykinin caused dose-dependent increases in tissue plasminogen activator antigen and activity (peak concentration  $31.8 \pm 3.4$  ng/mL and  $21.9 \pm 7.6$  IU/mL, respectively;  $P < 0.001$ ) and estimated antigen and activity release (peak release  $152 \pm 46$  ng per 100 mL/min and  $154 \pm 22$  IU/100 mL/min, respectively;  $P < 0.005$ ). Compared with placebo, thiorphan augmented bradykinin-mediated vasodilatation (1.4-fold;  $P < 0.0001$ ) and net tissue plasminogen activator release (1.5-fold;  $P < 0.005$ ). Neutral endopeptidase contributes to bradykinin metabolism in heart failure patients maintained on angiotensin converting enzyme inhibitor therapy. Our findings may explain some of the clinical effects of combined angiotensin-converting enzyme and neutral endopeptidase inhibition, including the greater vasodepressor effect observed with combined therapy when compared with angiotensin converting enzyme inhibition alone. (*Hypertension*. 2004;44:913-918.)

**Key Words:** heart failure ■ angiotensin-converting enzyme ■ bradykinin ■ endothelium

Bradykinin is a potent endothelium-dependent vasodilator peptide released at sites of inflammation and coagulation. Apart from vasodilatation, it also stimulates endothelial release of the prolytic factor tissue-type plasminogen activator (t-PA), and these effects are mediated by the constitutively expressed B<sub>2</sub> receptor.<sup>1,2</sup> Removal of the C-terminal arginine from bradykinin results in formation of des-Arg<sup>9</sup>-bradykinin, the principal ligand for the B<sub>1</sub> kinin receptor in plasma.<sup>3</sup> However, the most potent endogenous ligand for the B<sub>1</sub> kinin receptor is Lys-des-Arg<sup>9</sup>-bradykinin.<sup>3</sup> The vascular B<sub>1</sub> receptor is normally expressed very weakly but is markedly upregulated in the presence of inflammation, cardiovascular disease, and angiotensin converting enzyme (ACE) inhibition,<sup>4</sup> where it also mediates vasodilatation.<sup>3</sup>

ACE is the principal enzyme responsible for the rapid turnover of bradykinin (plasma half life  $\approx$  15 seconds) and its

metabolites.<sup>3</sup> It has been widely established that ACE inhibitors improve morbidity and mortality in patients with heart failure,<sup>5</sup> and these benefits may be due, at least in part, to inhibition of bradykinin metabolism.<sup>6,7</sup> ACE inhibition increases the plasma half lives of bradykinin and des-Arg<sup>9</sup>-bradykinin  $\approx$  9 and 2-fold, respectively,<sup>8</sup> and at a functional level, potentiates the vascular actions of bradykinin in the human forearm<sup>1,9</sup> and coronary<sup>10</sup> circulations. Moreover, bradykinin antagonism causes vasoconstriction<sup>7</sup> and attenuates the fall in blood pressure<sup>6</sup> in patients treated with ACE inhibitor therapy.

Neutral endopeptidase (EC 3.4.24.11; neprilysin [NEP]) is a membrane bound metallopeptidase that colocalizes with ACE and metabolizes a number of vasodilator and vasoconstrictor peptides, including atrial natriuretic peptide, substance P, endothelin-1, and bradykinin.<sup>11</sup> NEP expression is

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upregulated in heart failure patients,<sup>12,13</sup> and in the presence of ACE inhibition, the contribution of NEP to bradykinin metabolism is increased.<sup>14</sup> NEP inhibition potentiates the half life of bradykinin<sup>15</sup> and augments bradykinin-mediated vasodilatation in vitro.<sup>16</sup> It is not known whether NEP inhibition augments the half life of Lys-des-Arg<sup>9</sup>-bradykinin. Indeed, it has been suggested that the Phe<sup>8</sup> residue may protect B<sub>1</sub> ligands from degradation by NEP.<sup>3</sup> Although the effects of NEP inhibition on systemic hemodynamics are variable,<sup>17–20</sup> clinical improvements have been reported during NEP inhibition in heart failure patients.<sup>21</sup>

Coadministration of ACE and NEP inhibitors may confer additional therapeutic efficacy. Combined ACE and NEP inhibition attenuates bradykinin degradation more effectively than either enzyme alone,<sup>22</sup> and in animal models, improves cardiac remodeling and survival to a greater extent than isolated ACE inhibition.<sup>23</sup> These cardioprotective effects are lost in transgenic mice lacking the B<sub>2</sub> kinin receptor.<sup>24</sup> In man, combined ACE and NEP inhibition reduces blood pressure to a greater extent than inhibition of either enzyme alone<sup>19,25</sup> and is associated with symptomatic and hemodynamic improvements in heart failure patients.<sup>26</sup> The hypothesis that combined ACE and NEP inhibition may improve symptoms and survival in heart failure patients to a greater extent than ACE inhibition alone has been evaluated recently in a large-scale clinical trial (Omapatrilat Versus Enalapril Randomized Trial of Utility in Reducing Events [OVERTURE]).<sup>27</sup>

We demonstrated previously that chronic ACE inhibition potentiates bradykinin-mediated vasodilatation and endothelial release of t-PA in forearm circulation of heart failure patients.<sup>1</sup> The aims of this study were to investigate whether local NEP inhibition augments the vascular actions of bradykinin and to examine the effects of B<sub>1</sub> receptor agonism in heart failure patients maintained on long-term ACE inhibitor therapy.

## Methods

### Patients

Ten patients with symptomatic heart failure and echocardiographic evidence of left ventricular systolic dysfunction attended, fasted at 9 AM on 2 occasions at least 2 weeks apart. The protocol was performed with the approval of the local ethics committee, in accordance with the Declaration of Helsinki and with the written informed consent of each patient. Patients were maintained on maximally tolerated ACE inhibitor therapy (ramipril 10 mg daily [*n*=4] and 5 mg daily [*n*=3]; enalapril 20 mg daily [*n*=1] and 10 mg daily [*n*=1]; and lisinopril 40 mg daily [*n*=1]) for at least 6 months before recruitment. On the morning of each visit, ACE inhibitor therapy was administered at 8 AM and diuretics were withheld.

### Measurements

Bilateral forearm blood flow was measured using venous occlusion plethysmography.<sup>1,7,9,28</sup> Heart rate and blood pressure were recorded in the noninfused arm using a semiautomated noninvasive oscillometric sphygmomanometer (Takeda UA 751; Takeda) at baseline and in the final minute of each drug infusion period, after forearm blood flow measurements and venous sampling.

Venous cannulae (17-gauge) were inserted bilaterally into a large antecubital vein. Throughout each study, 10 mL of blood was collected from each arm into acidified buffered citrate (Biopool Stabilyte; Umeå; for t-PA assays) and citrate (Monovette, Sarstedt,

Numbrecht; for plasminogen activator inhibitor-1 [PAI-1] assays). Platelet-free plasma was prepared as described previously<sup>1,28</sup> and stored at –80°C before assay. Plasma concentrations of t-PA and PAI-1 antigen were determined using an ELISA, t-PA activity using a photometric method,<sup>28</sup> and ACE activity using colorimetric spectrophotometry (reference range 8 to 55 U/L; Sigma).<sup>29</sup>

### Study Protocol

A 27-gauge steel needle was inserted into the brachial artery of the nondominant arm. After 15 minutes equilibration with an infusion of 0.9% saline, patients were randomized to receive an intrabrachial infusion of thiorphan (30 nmol/min; Clinalfa AG) or saline placebo for 3 hours. The dose of thiorphan was chosen based on previous forearm studies<sup>30</sup> to achieve a local plasma concentration >10-fold the IC<sub>50</sub> of thiorphan for NEP in vitro.<sup>31</sup> Thiorphan or placebo was coinfused with bradykinin (30, 100, and 300 pmol/min; Clinalfa AG),<sup>7</sup> Lys-des-Arg<sup>9</sup>-bradykinin (1, 3, and 10 nmol/min; Clinalfa AG), atrial natriuretic peptide (10, 30, and 100 pmol/min; Clinalfa AG),<sup>32</sup> and sodium nitroprusside (2, 4, and 8 µg/min; David Bull Laboratories)<sup>28</sup> for 10 minutes at each dose. There was a 20 minute washout infusion of 0.9% saline between compounds. The order of infusion was randomized between patients but was maintained for both visits. The doses of Lys-des-Arg<sup>9</sup>-bradykinin were chosen based on binding affinity data<sup>33</sup> and the hypotensive dose response in rodents and nonhuman primates.<sup>34,35</sup> The combined rate of infusion remained constant throughout each study at 1 mL/min.

### Data Analysis and Statistics

Plethysmographic data were extracted from Chart data files, and forearm blood flow was calculated as described previously.<sup>1,28</sup> Estimated net release of t-PA antigen and activity were calculated as the product of infused forearm plasma flow and the t-PA concentration difference between the infused and noninfused arms.<sup>1,28</sup> Statistical analyses were performed using ANOVA or, where appropriate, paired *t* tests.

## Results

There were no significant differences in heart rate, blood pressure, or baseline forearm blood flow during or between study days (Table 1). Consistent with previous studies,<sup>2</sup> 1 subject developed transient upper limb edema with 300 pmol/min of bradykinin that rapidly resolved on cessation of the infusion. There were no other reported side effects.

### Plasma ACE Activity

Baseline plasma ACE activity was similar between thiorphan and placebo study visits (12.3±2.6 versus 9.7±1.5 U/mL, respectively; *P*=0.7). Compared with baseline, there were no significant differences in plasma ACE activity measured after 90 minutes of thiorphan (10.7±2.5 U/mL; *P*=0.3) or placebo (10.2±1.7 U/mL; *P*=0.3) coinfusion.

### Forearm Blood Flow Responses

Bradykinin, atrial natriuretic peptide, and sodium nitroprusside caused dose dependent increases in forearm blood flow in all studies (*P*<0.0001 for all; Figure 1). Forearm blood flow did not change during Lys-des-Arg<sup>9</sup> bradykinin infusion (Figure 1). Compared with saline placebo, coinfusion of thiorphan augmented forearm vasodilatation to bradykinin (*P*<0.0001; Figure 1) but not atrial natriuretic peptide or sodium nitroprusside.

### Plasma Fibrinolytic Factors

There were no significant differences in baseline plasma t-PA antigen (10.8±0.9 versus 9.8±0.9 ng/mL), t-PA activity



**TABLE 1. Patient Characteristics and Baseline Hemodynamics (n=10)**

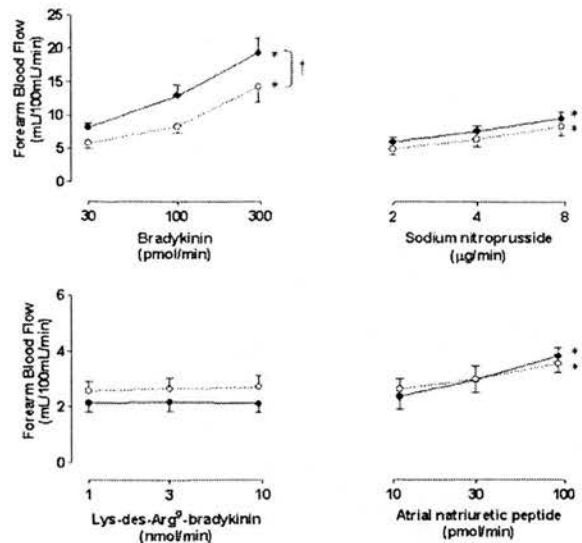
Variables	All Patients (n=10)	Units
Age (range)	64 (52–73)	Years
Gender (male/female)	5/5	n
Diagnosis (IHD/DCM)	8/2	n
NYHA class (II/III)	6/4	n
Risk factors for IHD		n
Smokers/nonsmokers	4/6	
Hypertension	4	
Diabetes mellitus	0	
Hypercholesterolemia	6	
Body mass index	30±1	kg/m <sup>2</sup>
Concomitant medications		n
ACE inhibitor	10	
Aspirin	7	
Diuretic	10	
β-blocker	8	
Statin	6	
Nitrate	1	
Digoxin	4	
Calcium antagonist	0	
Spironolactone	2	
Warfarin	3	
Amiodarone	2	
Echocardiography		
Ejection fraction	31±2	%
LVEDD	61±3	mm
Baseline heart rate		bpm
Thiorphan visit	58±3	
Placebo visit	56±2	
Baseline mean arterial pressure		mm Hg
Thiorphan visit	92±5	
Placebo visit	86±4	
Baseline forearm blood flow		mL per 100 mL/min
Thiorphan visit	2.0±0.4	
Placebo visit	1.9±0.2	

Data are expressed as No. of patients or mean±SEM.

Mean arterial pressure was calculated as diastolic pressure plus one third pulse pressure.

IHD indicates ischemic heart disease; DCM, idiopathic dilated cardiomyopathy; NYHA, New York Heart Association; LVEDD, left ventricular end diastolic diameter.

(0.1±0.1 versus 0.4±0.1), or PAI-1 antigen concentrations (37.3±3.7 versus 38.4±6.2 ng/mL) between thiorphan and placebo, respectively. Bradykinin caused a dose-dependent increase in plasma t-PA antigen and activity concentrations in the infused arm ( $P<0.001$  for all; Table 2) and the net release of t-PA antigen and activity in all studies ( $P<0.005$  for all; Figure 2). Compared with placebo, thiorphan augmented the increase in plasma t-PA activity concentration (21.9±2.4 versus 24.8±2.6 IU/mL, respectively at bradykinin, 300 pmol/min;  $P<0.05$ ; Table 2) in the infused arm and the net release of t-PA antigen (157±46 versus 233±46 ng per 100



**Figure 1.** Effect of thiorphan (●) and placebo (○) on infused forearm blood flow during intra-arterial infusion of bradykinin, sodium nitroprusside, atrial natriuretic peptide, and Lys-des-Arg<sup>9</sup>-bradykinin (\* $P<0.0001$ , ANOVA dose response; † $P<0.0001$ , ANOVA thiorphan vs placebo).

mL/min, respectively at bradykinin, 300 pmol/min;  $P<0.005$ ; Figure 2) and activity (155±22 versus 244±51 ng per 100 mL/min, respectively at bradykinin, 300 pmol/min;  $P<0.005$ ; Figure 2). There was a trend toward an increase in t-PA antigen in the infused arm during thiorphan infusion compared with placebo (32.5±3.4 versus 36.5±4.2 ng/mL, respectively at bradykinin, 300 pmol/min;  $P=0.058$ ; Table 2). Because of systemic overspill, bradykinin increased plasma t-PA antigen and activity concentrations in the noninfused arm ( $P<0.01$  and  $P<0.05$ , respectively; Table 2) that, for t-PA activity, was greater during thiorphan infusion ( $P<0.05$ ; Table 2). There were no significant changes in t-PA antigen, activity, or net t-PA release during infusion of atrial natriuretic peptide or Lys-des-Arg<sup>9</sup>-bradykinin.

There was a significant reduction in plasma PAI-1 antigen concentrations in the infused arm during bradykinin coinfusion with thiorphan (baseline 33.3±3.5 versus 29.4±3.4 ng/mL at bradykinin, 300 pmol/min;  $P<0.05$ ) but not placebo. There were no significant changes in PAI-1 antigen concentration during infusion of Lys-des-Arg<sup>9</sup>-bradykinin or atrial natriuretic peptide.

## Discussion

For the first time, we demonstrated that acute local NEP inhibition augments bradykinin-mediated endothelium-dependent vasodilatation and endogenous t-PA release in heart failure patients maintained on chronic ACE inhibitor therapy. In addition, this is the first clinical study to show that the B<sub>1</sub> receptor does not mediate vasodilatation or endothelial t-PA release in these patients. Our findings support the hypothesis that bradykinin may contribute to the systemic hemodynamic differences observed between combined ACE and NEP inhibition, and ACE inhibition alone.

## Clinical Implications

There is now substantial evidence that bradykinin contributes to the systemic hemodynamic and anti-ischemic effects of

**TABLE 2. Effect of Thiorphan and Placebo on Forearm Blood Flow (FBF) and Plasma Tissue Plasminogen Activator (t-PA) Antigen and Activity Concentrations in the Infused and Noninfused Forearms During Coinfusion of Bradykinin, Atrial Natriuretic Peptide, and Lys-des-Arg<sup>9</sup>-Bradykinin**

Parameter	Bradykinin pmol/min				Atrial Natriuretic Peptide pmol/min				Lys-des-Arg <sup>9</sup> -Bradykinin nmol/min			
	Baseline	30	100	300	Baseline	10	30	100	Baseline	1	3	10
<b>Thiorphan infusion</b>												
FBF, mL per 100 mL/min												
Infused	2.1±0.2	8.2±0.7	13±1.5	19±2.2*†	2±0.3	2.4±0.4	3±0.4	3.8±0.6*	2.2±0.3	2.1±0.3	2.2±0.3	2.1±0.3
Noninfused	1.5±0.3	2±0.4	1.9±0.3	1.7±0.3	1.4±0.2	1.5±0.3	1.6±0.3	1.7±0.3	1.6±0.3	1.5±0.3	1.5±0.3	1.6±0.3
t-PA antigen, ng/mL												
Infused	9.3±0.8	18.0±1.8	26.2±2.9	36.5±4.2*	10.8±0.9	10.7±0.8	10.2±0.7	9.5±0.8	10.1±0.9	10.2±0.9	10.1±0.8	9.8±0.8
Noninfused	9.6±0.7	10.8±1	12.9±1	17.6±2.5*	9.9±0.8	10.4±0.9	10.3±0.9	9.8±0.8	10.2±0.9	10.2±0.9	10±0.8	9.6±0.8
t-PA activity, IU/mL												
Infused	0.1±0.1	8.7±1.4	16.2±2.4	24.8±2.6*\$	1.3±0.5	1.1±0.4	0.8±0.4	0.5±0.2	0.8±0.4	0.8±0.5	0.5±0.3	0.4±0.2
Noninfused	0.1±0.1	1.0±0.2	3.1±1	6±1.2†\$	0.6±0.2	0.7±0.3	0.5±0.3	0.4±0.3	0.7±0.3	0.2±0.2	0.4±0.3	0.3±0.2
<b>Placebo infusion</b>												
FBF, mL per 100 mL/min												
Infused	2±0.3	5.8±0.8	8.3±0.9	14.4±2.2*†	2.2±0.4	2.6±0.4	3±0.5	3.6±0.6*	2.5±0.3	2.6±0.3	2.7±0.4	2.7±0.4
Noninfused	1.9±0.3	2.1±0.4	2±0.3	2.1±0.4	2±0.4	2±0.3	2±0.3	2±0.3	1.8±0.2	1.9±0.3	1.9±0.3	1.9±0.3
t-PA antigen, ng/mL												
Infused	9.1±0.7	14.2±1	22.8±2.2	32.5±3.4*	9.8±0.8	9.4±0.7	9.2±0.7	9.5±0.8	9.6±0.8	9.7±0.8	9.5±0.8	9.4±0.8
Noninfused	9.3±0.8	9.8±0.7	11.2±0.8	14.6±1.2*	10±0.7	9.8±0.6	10±0.7	9.6±0.7	9.4±0.8	9.3±0.8	9.2±0.7	9±9.7
t-PA activity, IU/mL												
Infused	0.3±0.1	4.6±1.3	12.6±2.4	21.9±2.4*\$	0.6±0.2	0.5±0.1	0.4±0.2	0.3±0.1	0.8±0.2	0.6±0.2	0.6±0.2	0.6±0.2
Noninfused	0.3±0.1	0.8±0.3	1.4±0.4	2.8±0.7†\$	0.2±0.1	0.3±0.1	0.2±0.1	0.1±0.1	0.3±0.1	0.3±0.1	0.3±0.1	0.3±0.2

ANOVA dose response \* $P<0.001$ , † $P<0.05$ ; ANOVA thiorphan vs placebo ‡ $P<0.001$ , § $P<0.05$ .

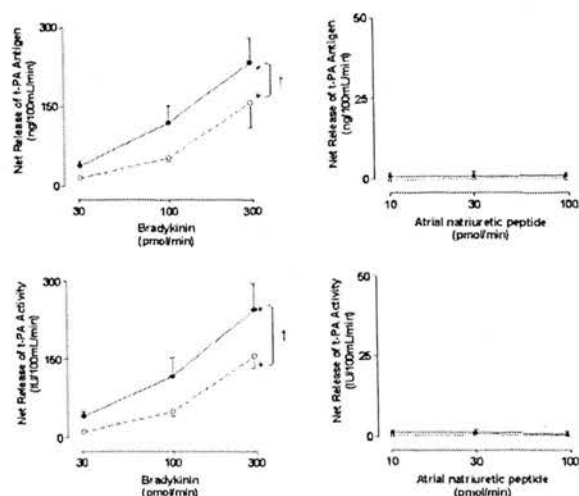
ACE inhibitor therapy.<sup>1,6,7,36</sup> Our findings suggest that bradykinin-mediated vasodilatation may contribute to the greater vasodepressor actions demonstrated with combined ACE and NEP inhibition compared with isolated ACE inhibition.<sup>19,25</sup> Moreover, despite the marked increase in bradykinin-induced t-PA release by ACE inhibition alone,<sup>1</sup> additional NEP inhibition causes further substantial augmentation of acute t-PA release. Together, these hemodynamic and profibrinolytic effects would be expected to have important therapeutic consequences. However, in the recent OVERTURE trial<sup>27</sup> of heart failure patients, treatment with omapatrilat, a combined ACE and NEP inhibitor, failed to reduce all-cause mortality when compared with enalapril, although it did reduce the combined secondary end point of cardiovascular death and hospitalization. Post hoc analysis redefining end points according to the Studies of Left Ventricular Dysfunction (SOLVD) criteria suggested that omapatrilat may be more effective at preventing cardiovascular events than enalapril, but that the additional benefit was substantially smaller than anticipated.<sup>27</sup> This may, in part, reflect the shorter duration of NEP inhibition compared with ACE inhibition with omapatrilat.<sup>37</sup> Given our findings, phar-

macological strategies offering a more balanced and prolonged duration of combined ACE and NEP inhibition may confer greater cardiovascular benefits.

Augmentation of bradykinin-mediated vasodilatation within the kidney may also contribute to the greater increase in renal blood flow observed with combined ACE and NEP inhibition than ACE inhibition alone.<sup>38</sup> As a result, it has been suggested that combined ACE and NEP inhibition may afford greater renal protection than ACE inhibition alone.<sup>38</sup> However, it should be noted that potentiating the vascular actions of kinins may have detrimental effects. Bradykinin has been implicated in the pathogenesis of ACE inhibitor-mediated angioedema.<sup>39</sup> An even greater incidence of angioedema has been reported after treatment with combined ACE and NEP inhibition.<sup>40</sup> Our findings are consistent with the suggestion that bradykinin may contribute to this rare but potentially life-threatening side effect.

### Role of B<sub>1</sub> Receptor

We demonstrated that intra-arterial Lys-des-Arg<sup>9</sup>-bradykinin, a potent and highly selective agonist at the human B<sub>1</sub> kinin receptor, has no effect on blood flow or endothelial t-PA



**Figure 2.** Effect of thiorphan (●) and placebo (○) on net tissue plasminogen activator (t-PA) antigen (top) and activity (bottom) release during intra-arterial infusion of bradykinin and atrial natriuretic peptide (\* $P < 0.005$ , ANOVA dose response; † $P < 0.005$ , ANOVA thiorphan vs placebo).

release in the forearm circulation of heart failure patients maintained on long-term ACE inhibitor therapy. This is in contrast to our previous findings that combined  $B_1$  and  $B_2$  receptor blockade, but not isolated selective blockade of the  $B_2$  receptor causes vasoconstriction in heart failure patients treated with ACE inhibition.<sup>7</sup> We infused Lys-des-Arg<sup>9</sup>-bradykinin at a dose that would achieve a local plasma concentration 20× greater than those shown previously to produce 50% of the maximal vasomotor response in human<sup>33</sup> and animal studies,<sup>34,35</sup> and therefore, our findings are unlikely to reflect an inadequate dose. Additional clinical studies using a selective  $B_1$  kinin receptor antagonist are now required to more fully investigate the role of the vascular  $B_1$  receptor in man.

### Atrial Natriuretic Peptide and Neutral Endopeptidase

Consistent with previous work demonstrating an impaired forearm vasodilator response to atrial natriuretic peptide in heart failure patients,<sup>32,41,42</sup> atrial natriuretic peptide caused a modest dose-dependent increase in forearm blood flow that was not augmented by local NEP inhibition. Although suppression of PAI-1 expression in endothelial cells has been reported *in vitro*,<sup>43</sup> we report for the first time that intra-arterial atrial natriuretic peptide does not directly alter either plasma PAI-1 or t-PA antigen concentrations *in vivo* in man.

Local NEP inhibition did not potentiate atrial natriuretic peptide-mediated forearm vasodilatation, in keeping with previous data demonstrating that intrabrachial thiorphan (30 nmol/min) does not increase endogenous plasma atrial natriuretic peptide concentrations in human forearm circulation.<sup>30</sup> At first, this may appear surprising given that systemic NEP inhibition augments plasma atrial natriuretic peptide concentrations in heart failure patients.<sup>21</sup> However, it is likely to reflect differences in the rate of clearance of atrial natriuretic peptide and bradykinin from forearm circulation. The half life of atrial natriuretic peptide (≈5 minutes) is greater than that of bradykinin (≈15

seconds). Assuming a transit time of the forearm vascular bed of ≈30 seconds, NEP inhibition with intrabrachial thiorphan is unlikely to result in sufficient local accumulation of atrial natriuretic peptide to augment forearm vasomotor responses. Moreover, the natriuretic peptide C receptor contributes equally to the clearance of plasma atrial natriuretic peptide, and this pathway is unaffected by NEP inhibition.

### Study Limitation

Although selective for NEP, thiorphan may cause some inhibition of ACE activity, and theoretically, our findings could represent further inhibition of ACE activity. Thiorphan exists as 2 enantiomers that, although equipotent for NEP inhibition, have differing potencies against ACE: selectivity of NEP compared with ACE inhibition of ≈50-fold for *S*-thiorphan and 200-fold for *R*-thiorphan.<sup>11</sup> The preparation of thiorphan used in this study contains equal proportions of both isomers.<sup>11</sup> We do not believe the effects of thiorphan were mediated through additional inhibition of ACE activity because local thiorphan infusion did not alter plasma ACE activity. Moreover, in a previous study<sup>30</sup> using the same dose of thiorphan, there were no effects on plasma angiotensin II concentrations in forearm circulation of healthy volunteers treated acutely with enalapril. However, we acknowledge that ACE inhibition by maximally tolerated doses of an ACE inhibitor may be incomplete,<sup>44</sup> and we cannot completely exclude a contribution of additional ACE inhibition to our study findings.

### Perspectives

We have demonstrated that local NEP inhibition augments bradykinin-mediated vasodilatation and endothelial t-PA release in heart failure patients maintained on long-term ACE inhibitor therapy. Using a potent  $B_1$  receptor agonist, we have shown that isolated stimulation of the  $B_1$  kinin receptor does not cause vasodilatation or endothelial t-PA release in man. These findings confirm that NEP contributes to bradykinin metabolism in heart failure patients and suggest that potentiation of the vascular and profibrinolytic actions of bradykinin may explain some of the observed effects in recent clinical trials of combined ACE and NEP inhibitor therapy.

### Acknowledgments

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**PUBLICATION 30**



# B1 Kinin Receptor Does Not Contribute to Vascular Tone or Tissue Plasminogen Activator Release in the Peripheral Circulation of Patients With Heart Failure

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**Objective**—Vascular expression of the B<sub>1</sub> kinin receptor is markedly upregulated with left ventricular dysfunction and angiotensin-converting enzyme (ACE) inhibition, but its function remains unclear. Inhibitors of ACE potentiate bradykinin-mediated B<sub>2</sub> receptor-dependent vasodilatation and tissue plasminogen activator (tissue-type plasminogen activator [t-PA]) release. We investigated the contribution of the B<sub>1</sub> receptor to the maintenance of vascular tone and t-PA release in patients with heart failure.

**Methods and Results**—Eleven patients were treated with enalapril (10 mg twice daily) or losartan (50 mg twice daily) in a randomized double-blind crossover trial. During week 6 of each treatment, patients received an intrabrachial infusion of Lys-des-Arg<sup>9</sup>-bradykinin (B<sub>1</sub> agonist; 1 to 10 nmol/min), bradykinin (30 to 300 pmol/min), Lys-[Leu<sup>8</sup>]-des-Arg<sup>9</sup>-bradykinin (B<sub>1</sub> antagonist; 1 to 10 nmol/min), and norepinephrine (60 to 540 pmol/min). Blood flow and t-PA release were measured using venous occlusion plethysmography and blood sampling. Bradykinin ( $P < 0.001$  for all), but not Lys-des-Arg<sup>9</sup>-bradykinin, caused vasodilatation and t-PA antigen and activity release. Norepinephrine ( $P < 0.001$ ), but not Lys-[Leu<sup>8</sup>]-des-Arg<sup>9</sup>-bradykinin, caused vasoconstriction. Compared with losartan, enalapril augmented bradykinin-mediated vasodilatation ( $P < 0.05$ ) and t-PA release ( $P < 0.01$  for all) but had no effect on B<sub>1</sub> receptor-mediated responses.

**Conclusions**—The B<sub>1</sub> kinin receptor does not have a major vasomotor or fibrinolytic role in patients with heart failure. Augmentation of kinin-mediated vasodilatation and t-PA release by ACE inhibition is restricted to the B<sub>2</sub> receptor. (*Arterioscler Thromb Vasc Biol.* 2005;25:772-777.)

**Key Words:** ACE inhibitors ■ bradykinin ■ heart failure ■ plasminogen activators ■ receptors

Bradykinin is the major effector for the kinin family of peptides in humans. It is released at sites of inflammation and coagulation and contributes to the systemic hemodynamic<sup>1,2</sup> and anti-ischemic<sup>3,4</sup> effects of angiotensin-converting enzyme (ACE) inhibitor therapy. Besides vasodilatation, bradykinin stimulates endothelial release of the pro-lytic factor, tissue plasminogen activator (t-PA), and these effects are mediated by the constitutively expressed B<sub>2</sub> kinin receptor.<sup>5</sup>

Des-Arg<sup>9</sup>-bradykinin is the principal ligand for the B<sub>1</sub> kinin receptor in human plasma and is generated by carboxypeptidases after removal of the C-terminal arginine from bradykinin. The vascular B<sub>1</sub> receptor is normally expressed very weakly but is markedly upregulated in the presence of inflammation,<sup>6</sup> ischemic left ventricular dysfunction,<sup>7</sup> cardiovascular disease,<sup>8</sup> and ACE inhibition.<sup>9</sup> In animal studies, stimulation of the B<sub>1</sub> receptor produces vasodilatation and a reduction in blood pressure.<sup>6,10–12</sup> Intense endothelial B<sub>1</sub> receptor expression has been demonstrated in atherosclerotic

human blood vessels,<sup>13</sup> and B<sub>1</sub> receptor stimulation induces dose-dependent vasodilatation in human coronary arteries in vitro.<sup>14</sup> Whether the B<sub>1</sub> receptor contributes to the vascular effects of kinins in vivo in humans remains unknown.

ACE is the principal enzyme responsible for the rapid breakdown of bradykinin (plasma half life ≈ 15 seconds) to its inactive metabolites.<sup>15</sup> In addition to increasing plasma bradykinin concentrations, ACE inhibition will favor bradykinin breakdown by alternative metabolic pathways including plasma carboxypeptidases, augmenting the generation of des-Arg<sup>9</sup>-bradykinin, and thereby potentiating both B<sub>1</sub> and B<sub>2</sub> receptor-mediated effects.<sup>15</sup> B<sub>2</sub> receptor antagonism attenuates the vasodepressor effect of a single oral dose of captopril in healthy volunteers and subjects with hypertension.<sup>2</sup> In patients with heart failure, the combined B<sub>1</sub> and B<sub>2</sub> kinin receptor antagonist, B9340, causes vasoconstriction in the forearm circulation in the presence, but not absence, of ACE inhibition,<sup>16</sup> and when administered systemically, it attenuates the hemodynamic effects of chronic ACE inhibition.<sup>1</sup>

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However, the role of the B<sub>1</sub> receptor in patients with heart failure and those treated with ACE inhibitor therapy remains to be established.

Although des-Arg<sup>9</sup>-bradykinin is the principal B<sub>1</sub> agonist present in plasma, it has only modest affinity for the human B<sub>1</sub> receptor and retains some activity at the human B<sub>2</sub> receptor.<sup>15</sup> Indeed, des-Arg<sup>9</sup>-bradykinin is only 100-fold more selective for the human B<sub>1</sub> receptor.<sup>15</sup> In contrast, Lys-des-Arg<sup>9</sup>-bradykinin, an endogenous tissue-based metabolite of kallidin, has ≈1000-fold greater affinity for the B<sub>1</sub> receptor than des-Arg<sup>9</sup>-bradykinin and is inactive at the B<sub>2</sub> receptor in humans.<sup>15,17,18</sup> Substitution of the Phe<sup>8</sup> residue in Lys-des-Arg<sup>9</sup>-bradykinin with Leu results in the formation of Lys-[Leu<sup>8</sup>]-des-Arg<sup>9</sup>-bradykinin, a synthetic peptide with potent selective inhibitory activity at the human B<sub>1</sub> receptor.<sup>6,15</sup>

Using custom-manufactured, clinical-grade preparations of Lys-des-Arg<sup>9</sup>-bradykinin and Lys-[Leu<sup>8</sup>]-des-Arg<sup>9</sup>-bradykinin, the aims of this study were to investigate whether the B<sub>1</sub> receptor contributes to the vascular actions of kinins in the patients with heart failure treated with ACE inhibition.

## Methods

This study was performed with the approval of the local research ethics committee in accordance with the Declaration of Helsinki and with the written informed consent of each subject.

## Drugs

Pharmaceutical grade bradykinin (Clinalfa AG, Läufelfingen, Switzerland), Lys-des-Arg<sup>9</sup>-bradykinin (Clinalfa), Lys-[Leu<sup>8</sup>]-des-Arg<sup>9</sup>-bradykinin (Clinalfa), HOE-140 (Clinalfa), B9340 (Clinalfa), and norepinephrine (Abbott Laboratories Ltd, Maidenhead, UK) were dissolved in physiological saline on the day of study. The doses of bradykinin and norepinephrine were chosen based on the results of previous studies.<sup>19</sup> The doses of Lys-des-Arg<sup>9</sup>-bradykinin and Lys-[Leu<sup>8</sup>]-des-Arg<sup>9</sup>-bradykinin were chosen based on the EC<sub>50</sub> (B<sub>1</sub> receptor, 0.2 nmol/L; B<sub>2</sub> receptor, >30 000 nmol/L) and IC<sub>50</sub> (B<sub>1</sub>, 1.3 nmol/L; B<sub>2</sub>, >30 000 nmol/L), respectively, for human kinin receptors *in vitro*,<sup>15</sup> data from human umbilical vein myography studies,<sup>18,20</sup> and the hypotensive dose response in rodents and nonhuman primates.<sup>6,11</sup>

## Human Umbilical Vein Studies

To confirm the efficacy of the B<sub>1</sub> receptor agonist, Lys-des-Arg<sup>9</sup>-bradykinin, and the B<sub>1</sub> antagonist, Lys-[Leu<sup>8</sup>]-des-Arg<sup>9</sup>-bradykinin, human umbilical cord was obtained from women aged 16 to 40 years undergoing routine cesarean section after uncomplicated pregnancy. Immediately after delivery, 10 cm umbilical cord was excised midway between placenta and child and placed in Krebs buffer solution (NaCl 6.954 mmol/L, KCl 4.7 mmol/L, CaCl<sub>2</sub> 2.5 mmol/L, MgSO<sub>4</sub> 1.17 mmol/L, NaHCO<sub>3</sub> 2 mmol/L, KH<sub>2</sub>PO<sub>4</sub> 1.18 mmol/L, EDTA 0.027 mmol/L, glucose 5.5 mmol/L; Fisher Scientific UK Ltd, Loughborough UK). Human umbilical vein was dissected into 3-mm rings as previously described.<sup>20</sup>

## Myography

Three hours after delivery, umbilical vein rings were mounted on wire myographs, suspended in organ baths containing 10 mL Krebs solution, and stretched with an initial tension of 2 g (Multimyograph System 700MO; JP Trading, Denmark). Krebs solution was maintained at 37°C and continually bubbled with 95% O<sub>2</sub>/5% CO<sub>2</sub>. Changes in tension were measured using an isometric transducer (Mac Laboratory 8; Analog Digital Instruments Pty Ltd, Australia).

After 60-minute equilibration, during which the tension was readjusted at 15-minute intervals, maximal contraction to KCl (60 mmol/L) was determined on 3 occasions, interspersed by 15-minute washout periods. Tissue rings were incubated with cap-

topril (1 μmol/L; Sigma Pharmaceuticals, St Louis, Mo) 30 minutes before performing cumulative dose responses to bradykinin (B<sub>2</sub> receptor agonist; 10<sup>-11</sup> to 10<sup>-6</sup> mol/L) and Lys-des-Arg<sup>9</sup>-bradykinin (B<sub>1</sub> receptor agonist; 10<sup>-11</sup> to 10<sup>-6</sup> mol/L) in the presence or absence of Lys-[Leu<sup>8</sup>]-des-Arg<sup>9</sup>-bradykinin (B<sub>1</sub> receptor antagonist; 1 μmol/L), HOE-140 (B<sub>2</sub> receptor antagonist; 1 μmol/L), or B9340 (combined B<sub>1</sub>/B<sub>2</sub> receptor antagonist; 1 μmol/L). Peptide antagonists were applied 10 minutes before agonists to ensure that equilibrium was obtained. Maximal contraction to 60 mmol/L KCl was determined on a final occasion at the end of each experiment. A single concentration curve for bradykinin and Lys-des-Arg<sup>9</sup>-bradykinin was obtained for each ring, and experiments were performed in parallel with rings from the same tissue.

## Immunohistochemistry

After contractile studies, human umbilical vein rings were fixed in formalin and embedded in paraffin wax. A hematoxylin and eosin stain was performed for each tissue section. B<sub>1</sub> and B<sub>2</sub> receptors were immunodetected using diaminobenzidine immunoprecipitation. Briefly, tissue was boiled in 0.01 mol/L sodium citrate (pH 6.0) for 8 minutes to facilitate antigen retrieval. Nonspecific binding sites were blocked with 1:100 donkey serum followed by 3% H<sub>2</sub>O<sub>2</sub>. Tissue sections were then incubated with the primary affinity purified goat polyclonal antibody specific for human B<sub>1</sub> or B<sub>2</sub> kinin receptor (Santa Cruz Biotechnology, Inc), followed by the secondary antibody (donkey anti-goat IgG; Santa Cruz Biotechnology Inc) or with the secondary antibody alone (control). Finally, tissues were treated with a peroxidase-antiperoxidase streptavidin-biotin conjugating system (Dako K0690) and visualized by conventional light microscopy using liquid diaminobenzidine precipitant (Dako K3465).

## Clinical Study

Eleven patients with symptomatic heart failure (New York Heart Association class II or III) and evidence of left ventricular systolic dysfunction (ejection fraction <40%, shortening fraction <20%, or left ventricular end diastolic dimension >55 mm) were recruited. Patients were maintained on maximally tolerated ACE inhibitor therapy for at least 6 months before enrollment. On study days, patients who attended fasted for 4 hours and diuretics were withheld for patient comfort.

When symptoms would allow, ACE inhibitor therapy was withdrawn for a period of 2 weeks (n=7). After this and in place of their usual ACE inhibitor, patients were randomized to receive 6 weeks of treatment with enalapril 10 mg<sup>21</sup> twice daily or losartan 50 mg<sup>22</sup> twice daily in a double-blind, crossover trial. One patient withdrew because of worsening symptoms and was replaced. During week 2 of ACE inhibitor withdrawal and weeks 6 and 12 of the crossover trial, patients attended on 2 occasions at least 3 days apart and underwent an agonist study on one occasion and an antagonist study on the other. The study order was randomized between patients but remained constant for each patient.

## Intra-Arterial Drug Administration

All studies were performed with patients lying supine in a quiet, temperature-controlled (22°C to 25°C) room. Under local anesthesia, a 27-gauge needle (Cooper Needle Works Ltd) was inserted into the brachial artery of the nondominant arm. The rate of intra-arterial drug infusion remained constant throughout at 1 mL/min.

## Forearm Blood Flow and Blood Pressure

Forearm blood flow was measured at 10-minute intervals in both arms using venous occlusion strain gauge plethysmography as previously described.<sup>16,23</sup> Heart rate and blood pressure were recorded in the noninfused arm at intervals throughout the study using a semi-automated noninvasive oscillometric sphygmomanometer (Takeda UA 751; Takeda Medical Inc, Japan).

## Venous Sampling and Assays

During agonist studies only, 17-gauge venous cannulae were inserted bilaterally into a large antecubital vein. Ten milliliters of blood were

withdrawn simultaneously from each arm at baseline and in the last minute of each drug infusion period and collected into acidified buffered citrate (Biopool Stabilyte, Umeå) for t-PA assays and citrate (Monovette, Sarstedt, Numbrecht) for plasminogen activator inhibitor (PAI) type-1 (PAI-1) assays. Samples were kept on ice before being centrifuged at 2000g for 30 minutes at 4°C. Platelet-free supernatant was decanted and stored at -80°C before assay. Plasma t-PA and PAI-1 antigen concentrations were determined using enzyme-linked immunosorbent assays and plasma t-PA activity by a photometric method.<sup>23</sup>

### Protocol Design

After 30-minute equilibration with 0.9% saline, patients received an intra-brachial infusion of Lys-des-Arg<sup>9</sup>-bradykinin (1, 3, and 10 nmol/min) and bradykinin (30, 100, and 300 pmol/min) on one occasion (agonist protocol), and Lys-[Leu<sup>8</sup>]-des-Arg<sup>9</sup>-bradykinin (1, 3, and 10 nmol/min) and norepinephrine (60, 180, and 540 pmol/min) on the other (antagonist protocol). Study drugs were infused in random order for 10 minutes at each dose and separated by a 20-minute infusion of 0.9% saline.

### Data Analysis and Statistics

Unless stated, all data are expressed as mean±SEM. Human umbilical vein responses are expressed as a percentage of the maximal contraction to 60 mmol/L KCl obtained at the end of each experiment. Plethysmographic data were extracted from chart data files and forearm blood flows were calculated as described previously.<sup>16</sup> Estimated net release of t-PA antigen and activity were defined as the product of the infused forearm plasma flow (based on the mean hematocrit and forearm blood flow) and the concentration difference between the infused and noninfused arms.<sup>3,23</sup> Statistical analyses were performed using analysis of variance (ANOVA) and statistical significance was taken at the 5% level.

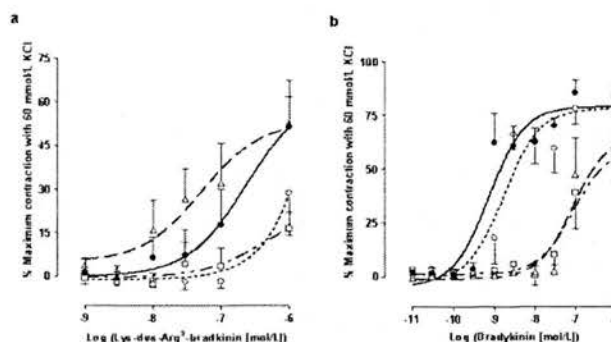
## Results

### Human Umbilical Vein Studies

Immunohistochemistry confirmed intense immunolabeling of both B<sub>1</sub> and B<sub>2</sub> receptors on human umbilical vein (Figure 1, available online at <http://atvb.ahajournals.org>). Consistent with previous work,<sup>18,20</sup> bradykinin and Lys-des-Arg<sup>9</sup>-bradykinin caused dose-dependent constriction of human umbilical vein rings (Figure 1;  $P<0.001$  for both). Lys-[Leu<sup>8</sup>]-des-Arg<sup>9</sup>-bradykinin and B9340 caused ≈10-fold rightward shift and HOE-140 caused a modest leftward shift in the dose response curve for Lys-des-Arg<sup>9</sup>-bradykinin (Figure 1a;  $P<0.001$ ,  $P<0.001$ , and  $P<0.05$ , respectively). In contrast, HOE-140 and B9340, but not Lys-[Leu<sup>8</sup>]-des-Arg<sup>9</sup>-bradykinin, caused a rightward shift in the dose response curve for bradykinin (Figure 1b;  $P<0.001$ ,  $P<0.001$ , and  $P=\text{not significant}$ , respectively).

### Clinical Study

Patients were predominantly male with mild to moderate congestive heart failure caused by ischemic heart disease (Table). There were no significant differences in heart rate, blood pressure, or baseline forearm blood flow during or between study days (Table I, available online at <http://atvb.ahajournals.org>). Compared with losartan, plasma ACE activity ( $42.2\pm11$  versus  $10.5\pm6.1$  U/L, respectively;  $P<0.05$ ) and angiotensin II concentrations ( $24.4\pm6.3$  versus  $7.8\pm1.6$  pg/mL, respectively;  $P<0.05$ ) were lower during treatment with enalapril.



**Figure 1.** Contraction of umbilical vein rings to (a) Lys-des-Arg<sup>9</sup>-bradykinin (black circles, solid line) and (b) bradykinin alone (black circles, solid line) or in the presence of Lys-[Leu<sup>8</sup>]-des-Arg<sup>9</sup>-bradykinin (1  $\mu\text{mol/L}$ ; white circles, fine dash), HOE-140 (1  $\mu\text{mol/L}$ ; white triangles, large dash), or B9340 (1  $\mu\text{mol/L}$ ; white square, intermittent dash). a, Lys-[Leu<sup>8</sup>]-des-Arg<sup>9</sup>-bradykinin and B9340 caused a rightward shift and HOE-140 caused a modest leftward shift in the dose response curve for Lys-des-Arg<sup>9</sup>-bradykinin ( $P<0.001$ ,  $P<0.001$ , and  $P<0.05$ , respectively; ANOVA). b, HOE-140 and B9340, but not Lys-[Leu<sup>8</sup>]-des-Arg<sup>9</sup>-bradykinin, caused a rightward shift in the dose response curve for bradykinin ( $P<0.001$ ,  $P<0.001$ , and  $P=\text{not significant}$ , respectively; ANOVA).

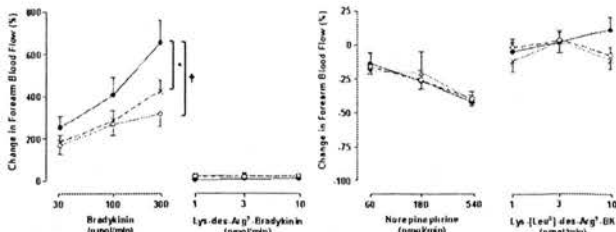
### Forearm Blood Flow

Bradykinin ( $P<0.0001$  for all), but not Lys-des-Arg<sup>9</sup>-bradykinin ( $P=\text{not significant}$  for all), caused dose-dependent vasodilatation in all studies (Figure 2). Bradykinin-mediated vasodilatation was augmented in

### Patient Characteristics (n=11)

Age, y (range)	68 (53–79)
Sex, male/female	10/1
Diagnosis, IHD/DCM	9/2
NYHA class, II/III	7/4
Risk factors for IHD, n	
Smokers/nonsmokers	2/9
Hypertension	3
Diabetes mellitus	0
Hypercholesterolemia	8
Body mass index, kg/m <sup>2</sup>	28±1
Concomitant medications, n	
ACE inhibitor	11
Aspirin	10
Diuretic	9
Beta-blocker	3
Statin	8
Nitrate	5
Digoxin	1
Calcium antagonist	4
Echocardiography	
Ejection fraction, %	36±3
LVEDD, mm	63±2

Data are expressed as number of patients or mean±SEM unless indicated. ACE indicates angiotensin-converting enzyme; DCM, idiopathic dilated cardiomyopathy; IHD, ischemic heart disease; LVEDD, left ventricular end diastolic diameter; NYHA, New York Heart Association.



**Figure 2.** Effect of bradykinin, Lys-des-Arg<sup>9</sup>-bradykinin, Lys-[Leu<sup>8</sup>]-des-Arg<sup>9</sup>-bradykinin, and norepinephrine on forearm blood flow in patients treated with enalapril (black circles), losartan (white circles), or after ACE inhibitor withdrawal (crosses). \* $P < 0.05$ , † $P < 0.005$  by ANOVA.

patients treated with enalapril compared with losartan ( $P < 0.005$ ; Figure 3) or ACE inhibitor withdrawal ( $P < 0.05$ ; Figure 2).

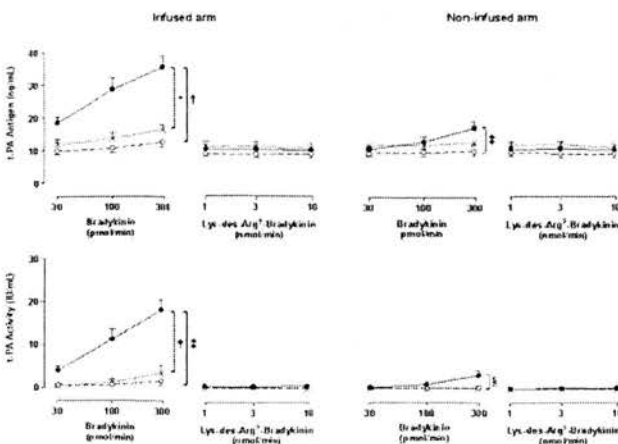
Norepinephrine ( $P < 0.0005$  for all), but not Lys-[Leu<sup>8</sup>]-des-Arg<sup>9</sup>-bradykinin ( $P = \text{not significant for all}$ ), caused dose-dependent vasoconstriction in all studies (Figure 2).

### Fibrinolytic Factors

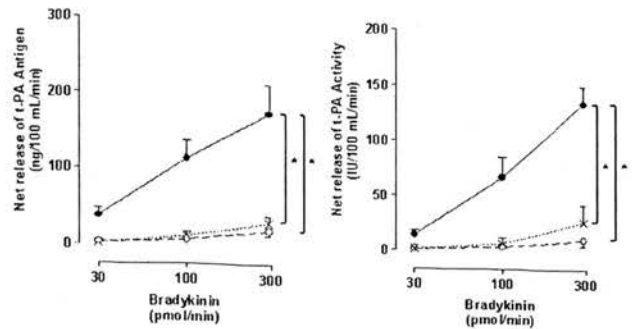
#### Release of t-PA

Bradykinin ( $P < 0.001$  for all), but not Lys-des-Arg<sup>9</sup>-bradykinin ( $P = \text{not significant for all}$ ), caused dose-dependent increases in plasma concentrations of t-PA antigen and activity in the infused arm and net release of t-PA antigen and activity in all studies (Figures 3 and 4). The bradykinin-mediated increase in plasma t-PA antigen and activity in the infused arm and net release of t-PA antigen and activity were augmented in patients treated with enalapril compared with losartan ( $P \leq 0.0005$  for all) or ACE inhibitor withdrawal ( $P < 0.005$  for all; Figures 3 and 4).

Consistent with systemic overspill,<sup>3</sup> bradykinin caused dose-dependent increases in plasma t-PA antigen and activity concentrations in the non-infused arm with enalapril therapy, and plasma t-PA antigen alone after ACE inhibitor withdrawal ( $P \leq 0.0001$  for all; Figure 3). Enalapril augmented the



**Figure 3.** Effect of bradykinin and Lys-des-Arg<sup>9</sup>-bradykinin on plasma t-PA antigen and activity concentrations in the infused and noninfused arm in patients treated with enalapril (black circles), losartan (white circles), or after ACE inhibitor withdrawal (crosses). \* $P < 0.005$ , † $P \leq 0.0005$ , ‡ $P < 0.0001$ , § $P < 0.01$  by ANOVA.



**Figure 4.** Effect of bradykinin on net release of t-PA antigen and activity in patients treated with enalapril (black circles), losartan (white circles), or after ACE inhibitor withdrawal (crosses). \* $P < 0.0001$  by ANOVA.

increase in t-PA compared with losartan therapy or ACE inhibitor withdrawal ( $P < 0.01$  for both; Figure 3).

### Plasma PAI-1 Antigen

There were no significant differences in basal PAI-1 antigen concentrations between study days (data on file). Consistent with an increase in PAI-1-mediated clearance after marked t-PA release,<sup>24</sup> and a potential time effect,<sup>25</sup> plasma PAI-1 antigen concentrations declined during infusion of bradykinin in both the infused ( $47.6 \pm 7.9$  ng/mL at baseline versus  $43.8 \pm 7$  ng/mL during bradykinin 300 pmol/min;  $P < 0.05$ ) and noninfused arms ( $51 \pm 8.2$  ng/mL at baseline versus  $44.5 \pm 7$  ng/mL during bradykinin 300 pmol/min;  $P < 0.05$ ) in patients treated with enalapril but not losartan or during ACE inhibitor withdrawal.

### Discussion

This is the first study to characterize the potential vasomotor and fibrinolytic role of the vascular B<sub>1</sub> kinin receptor in vivo. We have demonstrated that the peptidic B<sub>1</sub> receptor agonist, Lys-des-Arg<sup>9</sup>-bradykinin, and antagonist, Lys-[Leu<sup>8</sup>]-des-Arg<sup>9</sup>-bradykinin, have no effect on vascular tone or endothelial t-PA release in the presence or absence of ACE inhibition. In contrast, and consistent with our previous unblinded and nonrandomized data,<sup>3</sup> ACE inhibition markedly augmented the vascular actions of bradykinin mediated via the B<sub>2</sub> receptor. We conclude that the B<sub>1</sub> receptor does not appear to have a major vasomotor or fibrinolytic role in the forearm circulation of patients with heart failure treated with chronic ACE inhibition.

Our findings are in contrast to previous in vitro and animal work demonstrating vasodilatation after B<sub>1</sub> receptor stimulation.<sup>6,9–12</sup> Before concluding that the B<sub>1</sub> kinin receptor does not mediate vasodilatation or endothelial t-PA release in the forearm circulation of patients with heart failure, we must first consider the following possibilities: the doses of the Lys-des-Arg<sup>9</sup>-bradykinin and Lys-[Leu<sup>8</sup>]-des-Arg<sup>9</sup>-bradykinin used in this study were inadequate; the custom-made peptides lacked biological activity; or the extent of ACE inhibition was insufficient to upregulate B<sub>1</sub> receptor expression.

We infused Lys-des-Arg<sup>9</sup>-bradykinin at a dose that was at least 20-fold greater than that previously shown to produce



50% of the maximal hypotensive response in both primate ( $EC_{50} \approx 0.1$  pmol/kg)<sup>6</sup> and rodent studies ( $EC_{50} \approx 0.3$  pmol/kg).<sup>11</sup> Similarly, Lys-[Leu<sup>8</sup>]-des-Arg<sup>9</sup>-bradykinin was infused at a dose 20-fold greater than that previously shown to abolish B<sub>1</sub> receptor-mediated vasomotor responses in animal models in vivo.<sup>6,11</sup> To address the issue of biological activity, we examined vasomotor responses to the custom-manufactured B<sub>1</sub> agonist and antagonist in isolated human umbilical vein.<sup>20</sup> The concentration-response curves obtained for Lys-des-Arg<sup>9</sup>-bradykinin and Lys-[Leu<sup>8</sup>]-des-Arg<sup>9</sup>-bradykinin were comparable with data from previous studies<sup>18,20</sup> and confirm efficacy at concentrations predicted to be achieved in the infused human forearm circulation.

Previous rodent studies have demonstrated that besides cardiovascular inflammation, chronic ACE inhibition upregulates functional vascular B<sub>1</sub> receptor expression.<sup>9</sup> We have examined B<sub>1</sub> receptor function in patients with heart failure treated with an effective evidence based dose of enalapril.<sup>21</sup> Moreover, plasma concentrations of angiotensin II and ACE activity confirmed significant inhibition of the renin-angiotensin system with enalapril at this dose. From our findings, therefore, we can conclude that the B<sub>1</sub> kinin receptor does not mediate vasodilatation or endothelial t-PA release in patients with mild to moderate heart failure treated with long-term ACE inhibitor therapy.

We have previously demonstrated that combined B<sub>1</sub> and B<sub>2</sub> receptor blockade, but not B<sub>2</sub> receptor blockade, causes peripheral vasoconstriction in patients with heart failure treated with ACE inhibition. In our current study, however, selective B<sub>1</sub> receptor antagonism had no effect on peripheral vascular tone. One potential explanation for this discrepancy is that the B<sub>1</sub> receptor may only mediate the vasomotor effects of kinins in the absence of B<sub>2</sub> receptor-mediated signaling. In support of this hypothesis, the B<sub>1</sub> and B<sub>2</sub> kinin receptors are coupled to similar G-protein subtypes and share the same intracellular signaling pathways.<sup>15</sup> In transgenic mice lacking the B<sub>2</sub> kinin receptor, the B<sub>1</sub> receptor is upregulated and assumes vascular functions normally associated with the B<sub>2</sub> receptor.<sup>26</sup> Moreover, consistent with these data, we have demonstrated that the B<sub>2</sub> receptor antagonist, HOE-140, augments the vasomotor responses to the B<sub>1</sub> agonist, Lys-des-Arg<sup>9</sup>-bradykinin, in human umbilical vein in vitro. Future studies examining the effects of B<sub>1</sub> receptor agonism and antagonism during concomitant administration of HOE-140 may help clarify the issue of kinin receptor cross-talk in the peripheral circulation of patients with heart failure.

### Study Limitations

It has been suggested that the extent of the inflammatory response in patients with congestive cardiac failure correlates with the severity of underlying heart failure.<sup>27</sup> We have examined B<sub>1</sub> receptor function in patients with mild to moderate (New York Heart Association class II or III) heart failure. We cannot exclude the possibility that vascular B<sub>1</sub> receptor expression may be restricted to patients with severe end-stage heart failure. In addition, the B<sub>1</sub> kinin receptor has been implicated in a number of alternative biological processes, including leukocyte trafficking<sup>28</sup> and ischemia-induced angiogenesis.<sup>29</sup> It remains possible that B<sub>1</sub> receptors,

mediating processes other than vasodilatation or endogenous fibrinolysis, may be present in the human forearm vasculature. Finally, we have examined B<sub>1</sub>-mediated responses in the forearm circulation of patients with heart failure. Specific vascular beds may differ in their response to B<sub>1</sub> agonists and the current findings cannot be extrapolated to the entire vasculature.

In conclusion, contrary to data from animal studies, we have demonstrated for the first time to our knowledge that the B<sub>1</sub> kinin receptor does not mediate vasodilatation or endothelial t-PA release in the peripheral circulation of patients with heart failure treated with long-term ACE inhibition. Our findings suggest that the beneficial vascular effects of ACE inhibitor therapy attributed to kinins are restricted to those mediated by the B<sub>2</sub> receptor and do not support a major role for the B<sub>1</sub> kinin receptor as a potential therapeutic target in patients with heart failure.

### Acknowledgments

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## **SECTION 5**

### **MISCELLANEOUS TOPICS AND REVIEWS (Publications 31-37)**

## **PUBLICATION 31**

# Local and Systemic Effects of Intra-arterial Desmopressin in Healthy Volunteers and Patients with Type 3 von Willebrand Disease

## Role of Interleukin-6

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### Key words

Plasminogen activators, desmopressin, von Willebrand factor, interleukin-6, factor VIII:C

### Summary

Intra-arterial desmopressin caused dose and time dependent increases ( $p < 0.001$  for all) in forearm blood flow (all doses) and plasma tissue plasminogen activator (t-PA) concentrations (desmopressin  $\geq 70$  ng/min). Although plasma t-PA concentrations rose in both forearms, there was a modest local release of t-PA in the infused forearm (14 ng/100 mL of tissue/min,  $p < 0.05$ ). At desmopressin doses  $\geq 300$  ng/min, plasma von Willebrand factor (vWf) and Factor VIII:C concentrations rose in both forearms ( $p < 0.001$ ) and correlated with the rise in interleukin-6 concentrations ( $r = 0.92$ ,  $p < 0.001$ ;  $r = 0.85$ ,  $p = 0.002$  respectively). Neither desmopressin nor substance P caused t-PA, vWf or Factor VIII:C release in the patients, although desmopressin increased plasma interleukin-6 concentrations as in healthy volunteers. We conclude that desmopressin releases t-PA, vWf and Factor VIII:C predominantly via systemic mechanisms, possibly mediated by cytokine release. Patients with type 3 vWD appear to have a generalised failure to release t-PA acutely despite a normal interleukin-6 response to desmopressin infusion.

### Introduction

Desmopressin (1-deamino-8-arginine vasopressin) increases plasma levels of factor VIII:C, von Willebrand factor (vWF) and tissue plasminogen activator (t-PA) in healthy volunteers and patients with mild haemophilia A and mild von Willebrand disease (vWD) (1, 2). Patients with severe (type 3) vWD are usually (3), but not universally (4), unresponsive to desmopressin. Although the precise mode of action of desmopressin remains incompletely understood, in vitro endothelial cell culture studies have suggested that desmopressin at very high concentrations induces endothelial vWF release by a mechanism which is dependent on the presence of peripheral blood monocytes (5), possibly

mediated by the production of a second messenger molecule (6). However, the results of such in vitro experiments, must be interpreted with caution, as they may fail accurately to reflect the function of the endothelium in vivo.

In addition to its haemostatic and fibrinolytic actions, desmopressin causes vasodilatation and, when administered systemically, may lead to facial flushing, an increase in heart rate and a fall in blood pressure. It has been hypothesised that the consequent systemic neurohumoral response to desmopressin induced vasodilatation may contribute to, or even mediate, the haemostatic and fibrinolytic response to desmopressin infusion (7). To avoid these confounding systemic effects, the use of locally active intra-arterial infusion of drugs can be used to investigate the acute local release of t-PA and vWf in the forearm circulation of man (8–14). We have previously been able to demonstrate that substance P, an endothelium- and neurokinin type 1 (NK<sub>1</sub>) receptor-dependent vasodilator (9), causes a selective dose-dependent release of t-PA from the human forearm without causing significant release of vWf or plasminogen activator inhibitor type 1 (PAI-1) despite prolonged infusions of up to 2 h (12, 13). In contrast, intra-arterial infusions of desmopressin have either failed to find (8), or shown substantial (11), local release of t-PA in the human forearm. The reasons for this apparent disparity, and the role of monocyte activation in this process, are unclear.

Endothelial cells are able to store and rapidly release active vWF multimers, especially after vascular injury (15). Increased plasma concentrations of both Factor VIII:C and vWf have been associated with chronic inflammatory conditions (16, 17) including atherosclerosis (18–20). Moreover, the synthesis and release of Factor VIII:C may be regulated by monokines and cytokines, such as interleukin-6 (IL-6) (21), which, in part, mediate the acute phase response (22). Given the apparent in vitro dependence of desmopressin induced vWf release on the presence of monocytes, we hypothesised that IL-6 may mediate, or contribute to, the haemostatic or fibrinolytic response to desmopressin infusion.

The aims of the present study were to assess the acute release of haemostatic (vWf and Factor VIII:C), fibrinolytic (t-PA and PAI-1) and cytokine (IL-6) factors within the forearm vascular bed in response to intra-arterial desmopressin using (a) an ascending dose design to define the dose at which systemic effects intervene, and then (b) prolonged infusions of a locally (70 ng/min) and a systemically (700 ng/min) active dose. In order to explore further the mechanism of haemostatic, fibrinolytic and cytokine factors release, the responses to intra-arterial substance P and desmopressin infusion were evaluated in two patients with severe type 3 vWD.

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## Methods

### Subjects and Patients

Sixteen healthy non-smoking men aged between 20 and 34 years and two male patients with type 3 vWD aged 26 and 45 years participated in the studies which were undertaken with the approval of the local research ethics committee and in accordance with the Declaration of Helsinki. The written informed consent of each subject was obtained before entry into the study. None of the subjects received vasoactive or non-steroidal anti-inflammatory drugs in the week before each phase of the study, and all abstained from alcohol for 24 h, and from food and caffeine-containing drinks for at least 5 h, before each study. All studies were performed in a quiet, temperature controlled room maintained at 22–24°C.

### Drugs and Intra-arterial Administration

The brachial artery of the non-dominant arm was cannulated with a 27-standard wire gauge steel needle (Cooper's Needle Works Ltd, Birmingham, UK) under 1% lignocaine (Xylocaine; Astra Pharmaceuticals Ltd, Kings Langley, UK) local anaesthesia and attached to a 16-gauge epidural catheter (Portex Ltd, Hythe, UK). Patency was maintained by infusion of saline via an IVAC P1000 syringe pump (IVAC Ltd, Basingstoke, UK). The total rate of intra-arterial infusions was maintained constant throughout all studies at 1 mL/min. Desmopressin (DDAVP® Ferring) and substance P (Clinalfa AG, Läufelfingen, Switzerland) were administered following dissolution in saline (0.9%; Baxter Healthcare Ltd, Thetford, UK).

### Forearm Blood Flow and Blood Pressure

Blood flow was measured in both forearms by venous occlusion plethysmography using mercury-in-silastic strain gauges applied to the widest part of the forearm (23, 24). During measurement periods the hands were excluded from the circulation by rapid inflation of the wrist cuffs to a pressure of 220 mmHg using E20 Rapid Cuff Inflators (D. E. Hokanson Inc, Washington, USA). Upper arm cuffs were inflated intermittently to 40 mmHg for 10 s in every 15 s to achieve venous occlusion and obtain plethysmographic recordings. Analogue voltage output from an EC-4 Strain Gauge Plethysmograph (D. E. Hokanson) was processed by a PowerLab® analogue-to-digital converter and Chart™ v3.5.7 software (AD Instruments Ltd, Castle Hill, Australia) and recorded onto a Macintosh PowerBook 520c computer (Apple Computers Inc, Cupertino, USA). Calibration was achieved using the internal standard of the plethysmograph.

Blood pressure was monitored in the non-infused arm at intervals throughout each study using a semi-automated non-invasive oscillometric sphygmomanometer (Takeda UA 751, Takeda Medical Inc, Tokyo, Japan) (25).

### Venous Sampling and Assays

Venous cannulae (17G) were inserted into large subcutaneous veins of the antecubital fossa in both arms as described previously (14). Fifteen ml of blood was withdrawn simultaneously from each arm and collected into acidified buffered citrate (Biopool® Stabilite™, Umeå, Sweden; for t-PA assays), citrate (Monovette®, Sarstedt, Nümbrecht, Germany; for PAI-1, vWf and Factor VIII:C assays) and ethylene diamine tetraacetic acid (Monovette®; for IL-6 assays and determination of haematocrit) tubes, and kept on ice before being centrifuged at 2,000 g for 30 min at 4°C. Platelet free plasma was decanted and stored at -80°C before assay (26).

Plasma PAI-1, t-PA and IL-6 antigen concentrations were determined using an enzyme-linked immunosorbent assay; Coaliza® PAI-1, Coaliza® t-PA (Chromogenix AB, Mölndal, Sweden) and Quantikine® HS (R & D Systems Europe, Abingdon, UK) respectively. Plasma PAI-1 and t-PA activities were determined by a photometric method, Coatest® PAI-1 and Coatest® t-PA (Chromogenix AB). Intra-assay coefficients of variation were 5.5%, 7.0% and 5.9% for t-PA, PAI-1 and IL-6 antigen, and 2.4% and 4.0% for t-PA and PAI-1 activity, respectively. Interassay coefficients of variability were 4.0%, 7.3%, 9.9%,

4.0% and 7.6% respectively. The sensitivities of the assays were 0.5 ng/ml, 2.5 ng/ml, 0.09 pg/mL, 0.10 IU/ml and 5 AU/ml respectively. Von Willebrand factor (vWf) antigen was determined using an enzyme-linked immunosorbent assay (Dako A/S, Glostrup, Denmark), and vWf activity by the measurement of platelet aggregation in response to ristocetin (ristocetin co-factor) (27), with assay sensitivities of 0.10 IU/mL and 0.10 IU/mL respectively. The intra-assay coefficients of variability were 5.2% and 6.6%, and interassay were 7.3% and <8% respectively. Factor VIII:C procoagulant activity was determined using a standard one stage assay on an ACL-3000+ coagulometer (Instrumentation Laboratory, Warrington, UK). Haematocrit was determined by capillary tube centrifugation and was obtained from the infused forearm at baseline and at the end of the study protocol.

### Study Design

Subjects and patients rested recumbent throughout each study. Strain gauges and cuffs were applied and the brachial artery of the non-dominant arm cannulated. Measurements of forearm blood flow were made between 3 and 6 min of each infusion period unless otherwise stated. Before participating in one of the following protocols, saline was infused for the first 30 min to allow time for equilibration, with forearm blood flow measured every 10 min and the final measurement taken as basal blood flow.

### Healthy Volunteer Studies: Protocol 1

Eight subjects received an incremental infusion of desmopressin at 10, 30, 100, 300 and 1000 ng/min for 6 min at each dose (total desmopressin dose of 13.08 µg) followed by a 20 min saline infusion. Venous blood samples were obtained from both forearms at baseline, during desmopressin infusion at 10, 100 and 1000 ng/min, and 20 min after cessation of the final desmopressin infusion of 1000 ng/min. Venous sampling was performed following blood flow measurements and necessitated extending the desmopressin infusion to 10 min at the 10, 100 and 1000 ng/min doses.

### Healthy Volunteer Studies: Protocol 2

Eight subjects received desmopressin infusion at 70 ng/min for 30 min followed by desmopressin at 700 ng/min for 30 min (total dose of 23.1 µg), before infusing saline for the final 60 min. Venous blood samples were obtained from both forearms at baseline, at 10, 20 and 30 min during each desmopressin infusion of 70 and 700 ng/min, and 20 and 60 min after cessation of the desmopressin infusion.

### Patient Study

The two patients with type 3 vWD (patient 1: vWf <0.10 IU/mL, ristocetin co-factor <0.10 IU/mL, Factor VIII:C 0.05 IU/mL; patient 2: <0.10 IU/mL, <0.10 IU/mL, 0.04 IU/mL respectively) received an infusion of substance P at 4, 8 and 16 pmol/min for 10 min at each dose (12) followed by 30 min infusions of saline and desmopressin at 700 ng/min (total dose of 21 µg), before a final infusion of saline for 60 min. Venous blood samples were obtained from both forearms before, at 10, 20 and 30 min during substance P and DDAVP infusions and 20 and 60 min after cessation of the desmopressin infusion. On completion of the study protocol, a bolus of Factor VIII concentrate (Haemate P, Centeon, Eastbourne, UK) was administered prior to removal of the cannulae in order to reduce the risk of bleeding.

### Data Analysis and Statistics

Plethysmographic data were extracted from the Chart™ data files and forearm blood flows were calculated for individual venous occlusion cuff inflations by use of a template spreadsheet (Excel v5.0; Microsoft Corporation, Cambridge, USA). Recordings from the first 60 s after wrist cuff inflation were not used because of the variability in blood flow responses that this causes (28). Usually, the last five flow recordings in each 3 min measurement period were calculated and averaged for each arm. Mean arterial pressure was defined as the



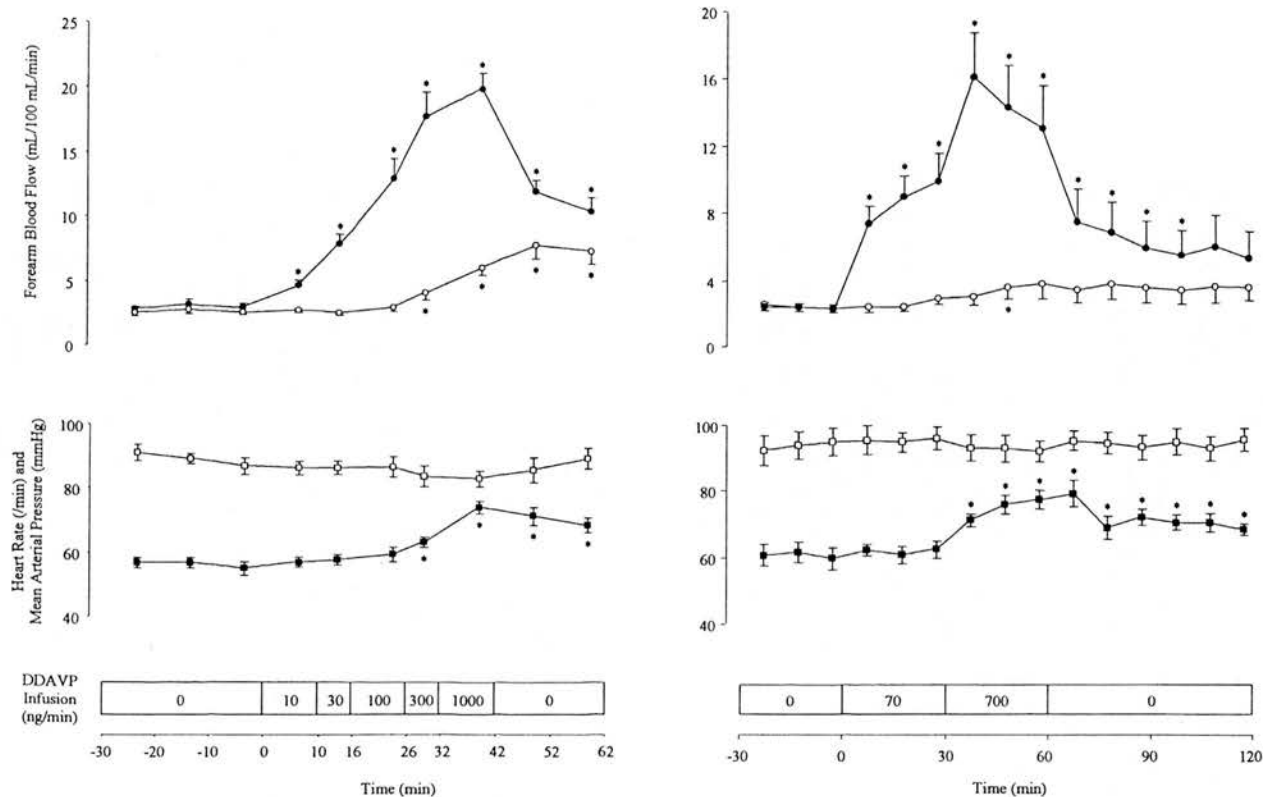


Fig. 1 Mean arterial pressure (open squares), heart rate (solid squares), and blood flow in the infused (solid circles) and non-infused (open circles) forearms during saline and desmopressin infusion. Protocol 1, left panel; protocol 2, right panel. One-way ANOVA:  $p < 0.001$  for all responses except mean arterial pressure, Two-way ANOVA:  $p =$  not significant (infused vs. non-infused forearms), paired t-test: \* $p < 0.05$  (vs. baseline)

sum of the diastolic blood pressure and a third of the pulse pressure. Estimated net release of t-PA activity and antigen was defined as the product of the infused forearm plasma flow (based on the haematocrit, Hct and the infused forearm blood flow, FBF) and the concentration difference between the infused ( $[t-PA]_{inf}$ ) and non-infused arms ( $[t-PA]_{non-inf}$ ).

$$\text{Estimated net t-PA release} = \text{FBF} \times \{1 - \text{Hct}\} \times \{[t-PA]_{inf} - [t-PA]_{non-inf}\}$$

Data were examined, where appropriate, by analysis of variance (ANOVA) with repeated measures, regression analysis and two tailed paired Student's t-test using Excel v5.0 (Microsoft). All results are expressed as mean  $\pm$  standard error of the mean. Statistical significance was taken at the 5% level.

## Results

Healthy volunteer and patient characteristics are shown in Table 1.

### Healthy Volunteer Studies: Protocol 1

Desmopressin infusion caused dose-dependent increases in blood flow in the infused forearm (Fig. 1; ANOVA,  $p < 0.001$ ) at all doses and in the non-infused forearm at doses  $\geq 300$  ng/min. Desmopressin also caused an increase in heart rate (ANOVA,  $p < 0.001$ ) but no fall in mean arterial pressure (ANOVA,  $p = 0.164$ ) at doses  $\geq 300$  ng/min (Fig. 1).

Plasma t-PA antigen and activity concentrations increased in a dose-dependent manner in both forearms (Fig. 2; ANOVA,  $p < 0.001$ ) during desmopressin infusion and correlated with changes in heart rate (infused arm;  $r = 0.46$ ,  $p = 0.003$  and  $r = 0.69$ ,  $p < 0.001$ : non-infused arm;  $r = 0.41$ ,  $p = 0.009$  and  $r = 0.64$ ,  $p < 0.001$  respectively) and non-infused forearm blood flow (infused arm;  $r = 0.45$ ,  $p = 0.003$  and  $r = 0.68$ ,  $p < 0.001$ : non-infused arm;  $r = 0.55$ ,  $p < 0.001$  and  $r = 0.72$ ,  $p < 0.001$  respectively). Overall, there were no significant differences in plasma

		Healthy Volunteers		Patients
		Protocol 1	Protocol 2	
Number		8	8	2
Age	(years)	24 $\pm$ 2	25 $\pm$ 2	36 $\pm$ 10
Height	(m)	1.78 $\pm$ 0.03	1.85 $\pm$ 0.02	1.76 $\pm$ 0.04
Weight	(kg)	80 $\pm$ 3	80 $\pm$ 2	86 $\pm$ 4
Body Mass Index	(kg/m <sup>2</sup> )	25 $\pm$ 1	23 $\pm$ 1	28 $\pm$ 0
Body Surface Area	(m <sup>2</sup> )	2.02 $\pm$ 0.04	1.98 $\pm$ 0.05	2.05 $\pm$ 0.07
Heart Rate	(/min)	57 $\pm$ 3	61 $\pm$ 3	61 $\pm$ 8
Blood Pressure	Systolic (mmHg)	139 $\pm$ 2	134 $\pm$ 6	146 $\pm$ 2
	Diastolic (mmHg)	67 $\pm$ 2	72 $\pm$ 5	87 $\pm$ 7
Haematocrit	Basal	0.398 $\pm$ 0.009	0.375 $\pm$ 0.007	0.398 $\pm$ 0.015
	Final	0.399 $\pm$ 0.010	0.376 $\pm$ 0.006	0.398 $\pm$ 0.015

Table 1 Healthy volunteer and patient characteristics

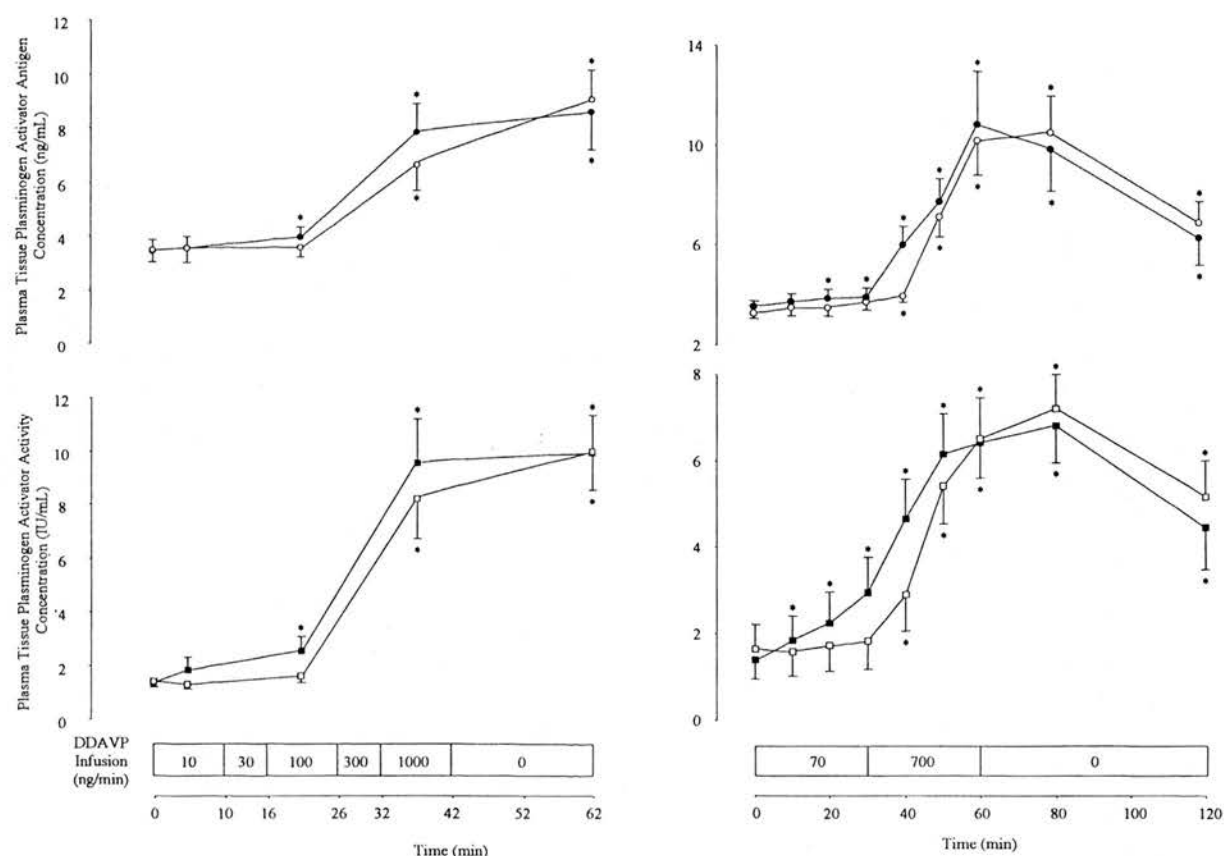


Fig. 2 Plasma t-PA antigen (circles) and activity (squares) concentrations in the infused (solid symbols) and non-infused (open symbols) forearms during saline and desmopressin infusion. Protocol 1, left panel; protocol 2, right panel. One-way ANOVA:  $p < 0.001$  for all responses, Two-way ANOVA:  $p$  = not significant (infused vs. non-infused forearms), Paired t-test: \* $p < 0.05$  (vs. baseline)

t-PA antigen and activity concentrations between the infused and non-infused arms during protocol 1 (two-way ANOVA,  $p = 0.654$  and  $0.361$  respectively). However, there was a small but significant increase in the plasma t-PA antigen concentration difference between the forearms (Table 2; ANOVA,  $p = 0.009$ ) and in the net local release of t-PA antigen and activity from the infused forearm (Table 2; ANOVA,  $p < 0.007$ ).

Plasma vWf and Factor VIII:C concentrations increased during, and 20 min following, the infusion of desmopressin at 1000 ng/min (Fig. 3; ANOVA,  $p < 0.001$ ). There were no significant differences in plasma vWf and Factor VIII:C concentrations between the infused and non-infused arms (two-way ANOVA,  $p = 0.853$  and  $0.595$ ) nor in the concentration difference between the forearms or the estimated net local release. The rise in plasma vWf and Factor VIII:C concentrations correlated with each other ( $r = 0.46$ ,  $p < 0.001$ ) and appeared to lag 20-30 min behind the increases in plasma t-PA concentrations.

There were no significant changes in plasma PAI-1 antigen and activity concentrations during protocol 1 (Table 2).

Plasma IL-6 concentrations increased in both forearms (Fig. 3; ANOVA,  $p < 0.001$ ) during, and 20 min following, the infusion of desmopressin at 1000 ng/min. The increase in plasma IL-6 concentrations appeared to be greater in the infused forearm but this was not statistically significant (two-way ANOVA,  $p = 0.126$  vs. non-infused forearm). Plasma IL-6 concentrations correlated with plasma vWf ( $r = 0.40$ ,  $p < 0.001$ ), Factor VIII:C ( $r = 0.24$ ,  $p = 0.030$ ), and t-PA antigen

( $r = 0.46$ ,  $p < 0.001$ ) and activity ( $r = 0.46$ ,  $p < 0.001$ ) concentrations. The time course of the rise in mean plasma IL-6 concentrations appeared to be similar to that for mean plasma vWf ( $r = 0.92$ ,  $p < 0.001$ ) and Factor VIII:C ( $r = 0.85$ ,  $p = 0.002$ ) concentrations.

#### Healthy Volunteer Studies: Protocol 2

Desmopressin infusion increased blood flow in the infused forearm (Fig. 1; ANOVA,  $p < 0.001$ ) at both doses and in the non-infused forearm at 700 ng/min (ANOVA,  $p = 0.028$ ). Compared with the blood flow after 10 min, the infused forearm blood flow was greater after 30 min of 70 ng/min of DDAVP (paired t-test;  $p = 0.010$ ) and lower after 30 min of 700 ng/min (paired t-test;  $p = 0.035$ ). There was an increase in heart rate (ANOVA,  $p < 0.001$ ) without alteration in mean arterial pressure during, and following, desmopressin infusion at 700 ng/min (Fig. 1).

Plasma t-PA antigen and activity concentrations increased in a time- and dose-dependent manner in both the infused and non-infused forearms (Fig. 2; ANOVA,  $p < 0.001$ ) during desmopressin infusion. Although increases occurred in the infused forearm at both doses, plasma t-PA concentrations rose in the non-infused forearm only during infusion of desmopressin at 700 ng/min. Overall, there were no significant differences in plasma t-PA antigen and activity concentrations between the infused and non-infused arms during the study (two-way ANOVA,  $p = 0.443$  and  $0.515$  respectively). However, there was a small but sig-

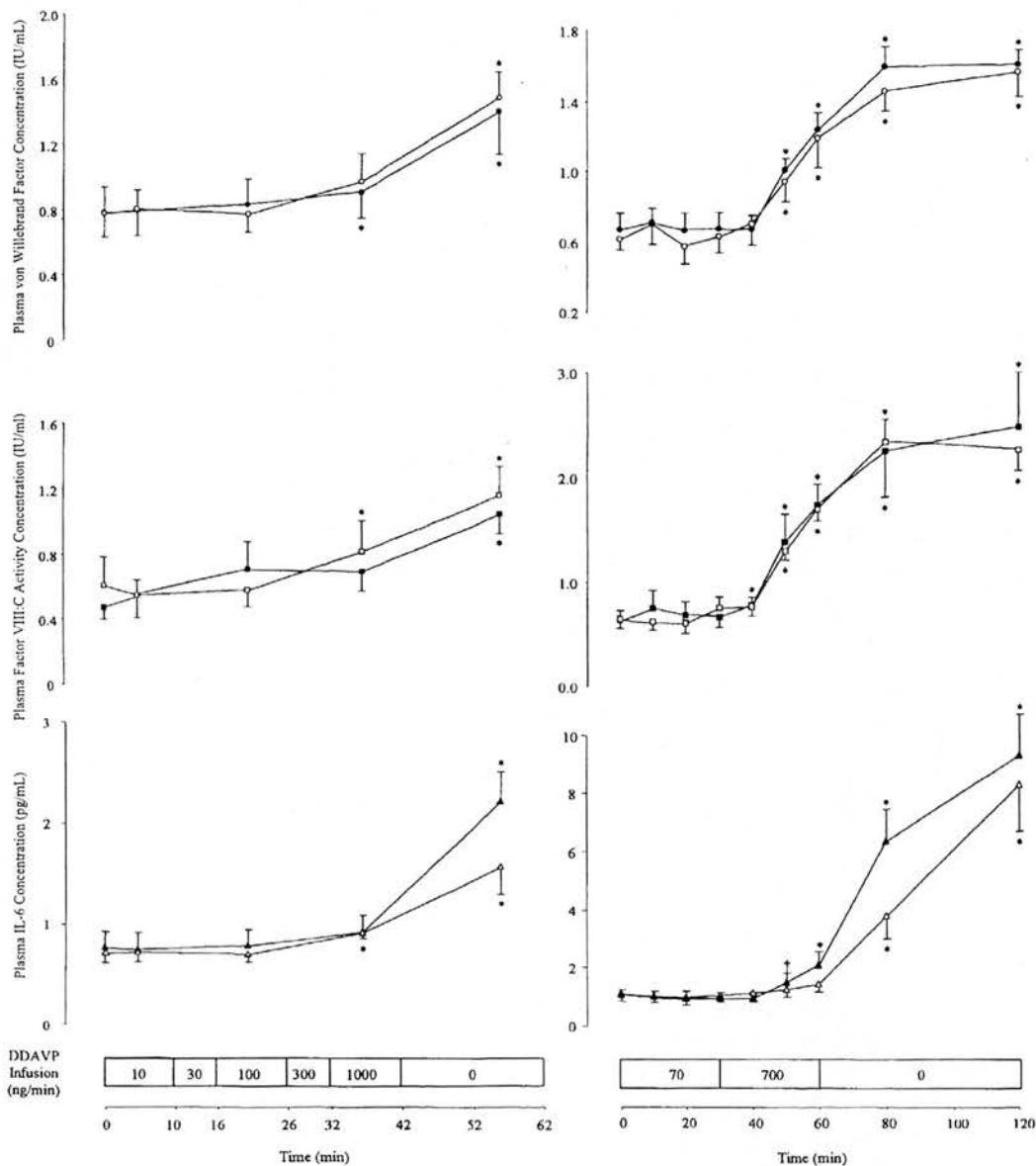


Fig. 3 Plasma von Willebrand factor (circles), Factor VIII:C (squares) and interleukin-6 (triangles) concentrations in the infused (solid symbols) and non-infused (open symbols) forearms during saline and desmopressin infusion. Protocol 1, left panel; protocol 2, right panel. One-way ANOVA:  $p < 0.001$  for all responses, Two-way ANOVA:  $p$  = not significant (infused vs. non-infused forearms), Paired t-test: \* $p < 0.05$ , † $p = 0.07$  (vs. baseline)

nificant increase in the plasma t-PA antigen and activity concentration difference between the forearms (Table 3; ANOVA,  $p = 0.037$  and  $p = 0.003$  respectively) and in the net local release of t-PA antigen and activity from the infused forearm (Table 3; ANOVA,  $p < 0.001$  for both).

Plasma vWf and Factor VIII:C concentrations increased during, and for up to 60 min following, the infusion of desmopressin at 700 ng/min (Fig. 3; ANOVA,  $p < 0.001$ ). There were no significant differences in plasma vWf and Factor VIII:C concentrations between the infused and non-infused arms (two-way ANOVA,  $p = 0.330$  and  $p = 0.650$  respectively) nor in the concentration difference between the forearms or the estimated net local release. Again, the rise in plasma vWf and Factor VIII:C concentrations were closely correlated with each other ( $r = 0.76$ ,  $p < 0.001$ ) and appeared to lag 20–30 min behind the increases in plasma t-PA concentrations.

There were no significant changes in plasma PAI-1 antigen and activity concentrations during protocol 2 although there was a trend for a reduction in plasma PAI-1 concentrations during the saline washout (Table 3).

Plasma IL-6 concentrations increased in both forearms (Fig. 3; ANOVA,  $p < 0.001$ ) during, and for up to 60 min following, the infusion of desmopressin at 700 ng/min. The increase in plasma IL-6 concentrations appeared to be greater in the infused forearm but this was not statistically significant (two-way ANOVA,  $p = 0.137$  vs. non-infused forearm). Plasma IL-6 concentrations correlated with plasma vWf ( $r = 0.59$ ,  $p < 0.001$ ), Factor VIII:C ( $r = 0.50$ ,  $p < 0.001$ ), and t-PA antigen ( $r = 0.43$ ,  $p < 0.001$ ) and activity ( $r = 0.24$ ,  $p = 0.004$ ) concentrations. The time course of the rise in mean plasma IL-6 concentrations appeared to be similar to that for plasma vWf ( $r = 0.87$ ,  $p < 0.001$ ) and Factor VIII:C ( $r = 0.84$ ,  $p < 0.001$ ) concentrations.

		Baseline	Desmopressin Infusion				Washout
Desmopressin (ng/min)		0	10	100	1000		0
Time (min)		0	10	26	42		62
t-PA Antigen	Concentration Difference Between Forearms (ng/mL)	-0.0 ± 0.1	0.0 ± 0.2	0.4 ± 0.1‡	1.2 ± 0.3‡		-0.4 ± 0.6 *
	Net Local (Infused) Forearm Release (ng/100 mL/min)	-0.0 ± 0.1	-0.1 ± 0.5	2.7 ± 0.9‡	13.5 ± 3.5‡		-4.4 ± 2.9 *
t-PA Activity	Concentration Difference Between Forearms (IU/mL)	-0.0 ± 0.1	0.5 ± 0.4	1.0 ± 0.4‡	1.4 ± 0.6‡		-0.1 ± 0.3 *
	Net Local (Infused) Forearm Release (IU/100 mL/min)	-0.1 ± 0.2	1.2 ± 1.0	6.4 ± 2.5‡	15.2 ± 6.5‡		-0.6 ± 1.7 *
PAI-1 Antigen (ng/mL)	Non-infused Arm	19 ± 4	19 ± 3	20 ± 5	17 ± 4		18 ± 5
	Infused Arm	16 ± 3	18 ± 3	19 ± 4	17 ± 4		16 ± 4
PAI-1 Activity (AU/mL)	Non-infused Arm	5 ± 2	9 ± 2	7 ± 2	5 ± 2		4 ± 2
	Infused Arm	5 ± 1	6 ± 1	5 ± 1	4 ± 1		5 ± 2

One-way ANOVA: \*p≤0.009, †p=0.056  
Paired t-test: ‡p<0.05 (vs baseline)

Table 2 Healthy Volunteer Studies: Protocol 1. Difference in forearm plasma tissue plasminogen activator (t-PA) concentrations and estimated net release of t-PA in the infused forearm, and plasma plasminogen activator inhibitor type 1 (PAI-1) antigen and activity concentrations in both forearms

Patient Studies

The study was well tolerated and there was no evidence of hematoma formation at the sites of arterial or venous puncture in either patient. In both patients, forearm blood flow appeared to increase only in the infused forearm with substance P infusion but in both forearms with desmopressin infusion (Fig. 4). Baseline plasma PAI-1 antigen and activity concentrations were 40 ± 10 ng/mL and 11 ± 2 AU/mL in the infused forearm, and 46 ± 8 ng/mL and 14 ± 3 AU/mL in the non-infused forearm respectively. Baseline plasma vWf and Factor VIII:C concentrations were <0.01 IU/mL and 0.05 IU/mL respectively in both forearms. Throughout saline, substance P and desmopressin infusions, there were no apparent changes in plasma t-PA (Fig. 5), PAI-1, vWf or Factor VIII:C concentrations. In contrast, plasma IL-6 concentrations ap-

peared to rise in both forearms following desmopressin but not substance P infusion.

Discussion

We have shown that, at locally active doses (70-100 ng/min), desmopressin causes a modest local release of t-PA in the forearm circulation without causing release of vWf or Factor VIII:C. At doses ≥300 ng/min, desmopressin causes systemic haemodynamic effects and marked increases in plasma t-PA, vWf and Factor VIII:C concentrations. These systemic increases in vWf and Factor VIII:C are closely associated with changes in plasma IL-6 concentrations. Indeed, despite the absence of a fibrinolytic or haemostatic response in patients with type 3 vWD, plasma IL-6 concentrations were also elevated following

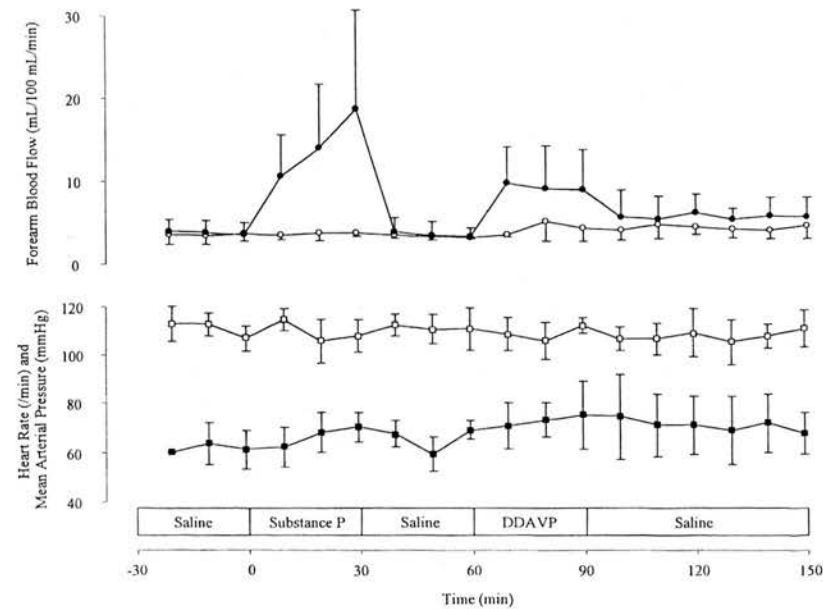


Fig. 4 Mean arterial pressure (open squares), heart rate (solid squares), and blood flow in the infused (solid circles) and non-infused (open circles) forearms during saline, substance P (4, 8 and 16 pmol/min for 10 min at each dose) and desmopressin (700 ng/min) infusion in two patients with type 3 von Willebrand disease

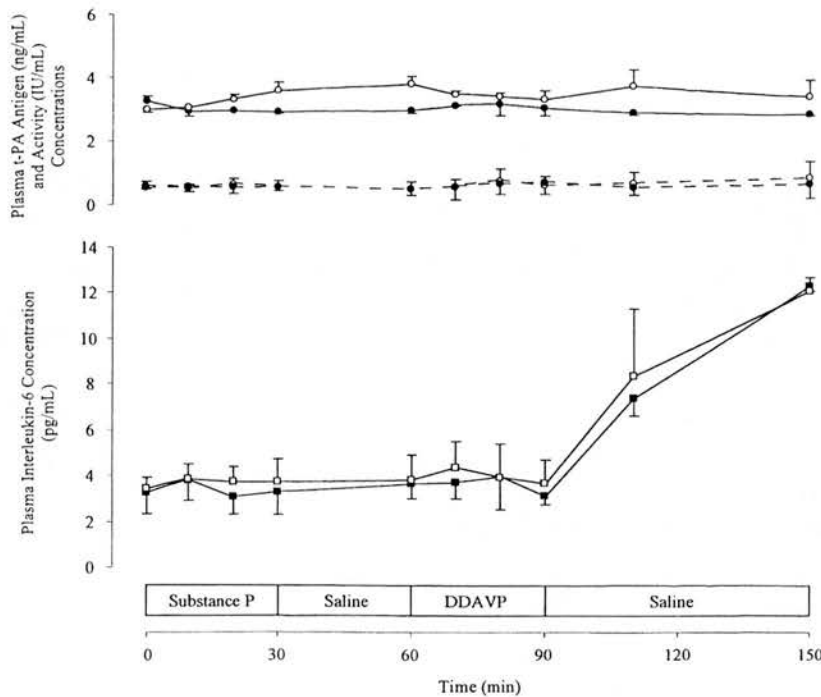


Fig. 5 Plasma tissue plasminogen activator (t-PA; circles) antigen (solid lines) and activity (dashed lines), and interleukin-6 (squares) concentrations in the infused (solid symbols) and non-infused (open symbols) forearms during saline, substance P (4, 8 and 16 pmol/min for 10 min at each dose) and desmopressin (700 ng/min) infusion in two patients with type 3 von Willebrand disease

desmopressin, but not substance P, infusion. We conclude that desmopressin appears to release t-PA, vWf and Factor VIII:C predominantly via systemic mechanisms which may, in part, be mediated by cytokine release. The modest local release of t-PA induced by desmopressin may be mediated by a direct action on the endothelium or indirectly via cytokines. Patients with type 3 vWD appear to have a generalised failure to release t-PA acutely despite mounting a normal IL-6 response to desmopressin infusion.

Our findings are at variance with those of Cash and colleagues (8) who reported an absence of an effect of intra-arterial desmopressin given at 100 ng/min for 10 min, although we detected a modest rise in plasma t-PA concentrations (14% for antigen). Euglobulin clot lysis, used by Cash, is a less sensitive method for detecting t-PA release and this may be the reason why his study failed to demonstrate an effect of local intra-arterial desmopressin infusion. In agreement with a more recent study (11), we find evidence of a modest local release of t-PA

Table 3 Healthy Volunteer Studies: Protocol 2. Difference in forearm plasma tissue plasminogen activator (t-PA) concentrations and estimated net release of t-PA in the infused forearm, and plasma plasminogen activator inhibitor type 1 (PAI-1) antigen and activity concentrations in both forearms

		Desmopressin 70 ng/min				Desmopressin 700 ng/min			Washout		
Time (min)		Baseline 0	10	20	30	40	50	60	80	120	
t-PA Antigen	Concentration Difference Between Forearms (ng/mL)	0.2 ± 0.1	0.3 ± 0.2	0.4 ± 0.2	0.2 ± 0.3	2.1 ± 0.7†	0.6 ± 0.4	0.7 ± 0.9	-0.7 ± 0.7	-0.6 ± 0.7	†
	Net Local (Infused) Forearm Release (ng/100 mL/min)	0.2 ± 0.1	1.5 ± 1.2	1.4 ± 1.2	0.7 ± 1.1	16.2 ± 4.7†	4.1 ± 3.3	1.1 ± 3.8	-3.3 ± 3.2	-3.3 ± 1.9	*
t-PA Activity	Concentration Difference Between Forearms (IU/mL)	-0.5 ± 0.3	0.0 ± 0.3	0.3 ± 0.4†	1.1 ± 0.5‡	1.8 ± 0.3‡	0.8 ± 0.2‡	-0.0 ± 0.5	-0.4 ± 0.5	-0.8 ± 0.7	†
	Net Local (Infused) Forearm Release (IU/100 mL/min)	-0.6 ± 0.3	0.7 ± 1.2	2.0 ± 1.9	6.0 ± 2.5‡	17.4 ± 3.9‡	7.3 ± 2.1‡	-0.6 ± 4.8	-3.1 ± 4.3	-2.7 ± 2.6	*
PAI-1 Antigen Non-infused Arm (ng/mL)		37 ± 13	36 ± 13	39 ± 15	33 ± 12	33 ± 12	32 ± 12	30 ± 11	28 ± 8	24 ± 7§	
Infused Arm		38 ± 13	41 ± 13	42 ± 12	40 ± 12	39 ± 12	37 ± 11	31 ± 11	27 ± 9	25 ± 7§	
PAI-1 Activity Non-infused Arm (AU/mL)		12 ± 3	13 ± 3	12 ± 3	12 ± 3	11 ± 3	11 ± 3	10 ± 3	5 ± 2‡	5 ± 2§	
Infused Arm		13 ± 4	10 ± 4	10 ± 4	10 ± 3	9 ± 3	10 ± 3	10 ± 3	8 ± 2‡	4 ± 1‡	

One-way ANOVA: \*p<0.001, †p<0.05  
 Paired t-test: ‡p<0.05, §0.10>p>0.05 (vs baseline)



during infusions of desmopressin at 70 and 100 ng/min. The estimated net local release of t-PA was 13.5 ng/100 mL of tissue/min which is similar to that previously reported by Wall and colleagues (11) 14.4 ng/100 mL of tissue/min.

Desmopressin induced vasodilatation and t-PA release appear to be time-dependent, with a slow onset and offset of action, even when confined to locally active doses (11). These properties of desmopressin are in marked contrast to those of other endothelium dependent stimulants such as substance P and bradykinin. In the human forearm, local infusions of substance P (12–14) and bradykinin (29) have a rapid onset and offset of action (<5 min) and, in comparison to desmopressin, are able to release 5–10-fold greater amounts of t-PA from the forearm (12, 29). Indeed, in marked contrast to desmopressin, systemic doses of substance P administered in the brachial artery of the forearm (12) cause a substantial local release of t-PA with wide and significant differences between the plasma t-PA concentrations of the infused and non-infused forearms. Although some of these differences may reflect the pharmacokinetics of the compounds, the infused forearm will have a local forearm concentration ~100-fold that of the systemic circulation and, in the presence of a directly acting endothelial stimulant, such high local concentrations should be reflected in substantial differences between the plasma concentrations of the infused and non-infused forearms. Substantial local release of t-PA did not occur with desmopressin infusion and the majority of the t-PA release occurred outwith the infused forearm.

In the present study, desmopressin caused vasodilatation at all doses used, t-PA release at doses  $\geq 70$  ng/min, and vWf and Factor VIII:C release at doses  $\geq 300$  ng/min. This apparent dose-dependence of responses to desmopressin may reflect a threshold effect on differing tissues and receptors. In ex vivo studies (30, 31), desmopressin causes a rapid onset and direct endothelium-dependent vasodilatation which is mediated through  $V_2$  receptors. However, desmopressin induced vWf release appears to be dependent on the presence of monocytes (5) and may be mediated by cytokines (22, 24). We, therefore, explored the relationship between IL-6, a marker of monocyte activation, and the blood flow, haemostatic and fibrinolytic responses to desmopressin infusion. We have demonstrated that plasma IL-6 concentrations rise in parallel with vWf and Factor VIII:C suggesting monocyte activation, possibly through IL-6, mediates the haemostatic response to desmopressin infusion.

Several studies have suggested that monocyte derived cytokines may have a role in modifying fibrinolytic and coagulation responses (32). Non-human primate studies indicate that IL-6 can significantly enhance the t-PA response to endotoxemia (33, 34). In human studies, infusion of tumor necrosis factor in healthy volunteers (35) and patients with cancer (36) is associated with marked increases in plasma t-PA concentrations. Although we have only measured IL-6 concentrations, it is equally possible that other, more rapidly mobilised, monocytic factors may play a role in mediating or augmenting the endothelial release of t-PA, vWf or Factor VIII:C. Moreover, although predominantly released by monocytes, IL-6 is released by other cell types and the observed effects may not be directly attributable to monocytes alone. Indeed, in contrast to forearm skeletal muscle, adipocytes (37) and adipose tissue (38) can release significant amounts of IL-6.

We have previously demonstrated that substance P causes an isolated increase in plasma t-PA concentrations without inducing vWf release (12). In the present study, the differing time course of t-PA and vWf release in response to desmopressin again indicates the presence of separate and distinct endothelial storage pools and release mechanisms for these factors. Impaired release of t-PA in response to venous occlu-

sion and desmopressin has been previously reported in patients with type 3 vWD (39–41). However, for the first time, we report the response to high, locally active, concentrations of intra arterial desmopressin and substance P in these patients. Despite different receptor mediated mechanisms of endothelial cell activation (9, 30, 31), neither desmopressin nor substance P caused t-PA, vWf or Factor VIII:C release in these patients. Interestingly, desmopressin infusion was associated with a similar increase in plasma IL-6 concentrations as with healthy volunteers, suggesting normal desmopressin induced monocyte activation but a generalised impairment in the release of endothelium derived haemostatic and fibrinolytic factors.

#### Acknowledgements

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**PUBLICATION 32**

## Editorial

# Intracoronary infusions and the assessment of coronary blood flow in clinical studies

Intracoronary drug administration may be desirable for a number of reasons and is used in therapeutic, diagnostic, interventional, and clinical research settings. One of the main indications for intracoronary drug administration is in the assessment of coronary blood flow either as a guide to intervention or as a clinical research tool. There are many methods of assessing coronary blood flow including the use of the angiogram derived corrected TIMI (thrombolysis in myocardial infarction) frame count<sup>1</sup> and the rate of decorrelation of the radiofrequency signal from intravascular ultrasound (IVUS) imaging catheters.<sup>2</sup> However, the most direct and widely used method of assessing coronary blood flow is the Doppler flow wire—a piezoelectric cell mounted on the tip of a 0.014 inch guide wire.<sup>3</sup>

The Doppler flow wire measures coronary blood flow velocity and, in order to measure coronary blood flow, knowledge of the cross sectional area of the vessel is required. The latter is usually estimated using quantitative coronary angiography (QCA), which assumes circular or elliptical luminal geometry, although greater accuracy can be obtained by using IVUS imaging catheters. Indices such as coronary flow reserve—the ratio of maximal to basal hyperaemic flow velocity—can be used to assess the functional severity of coronary stenoses and the dynamic integrity of the microcirculation without determining luminal cross sectional area. However, fractional flow reserve, which is measured using a pressure wire (guide wire with ability to measure distal coronary artery pressure), is increasingly being used to determine the functional severity of coronary stenoses since it is more reproducible, lesion specific, and less dependent on systemic haemodynamic parameters.<sup>4,6</sup>

Clinical research studies assessing coronary vasomotor responses to drug infusion have used endothelium

dependent and independent vasodilators as well as agonists and antagonists of physiological mediators (table 1). The magnitude and variability of coronary responsiveness is highly dependent on the agent used and crucially on the method of measurement and the mode of administration.

### Assessment of coronary vasomotor responses: QCA or IVUS

Although well established, QCA has many limitations in the measurement of vasomotor and blood flow responses.<sup>7,8</sup> It tends to underestimate the luminal area<sup>9</sup> and functional severity of coronary stenoses.<sup>7</sup> Contrast agent injection not only necessitates the interruption of the drug infusion and aspiration of the catheter,<sup>10</sup> but may itself be vasoactive and directly alter coronary blood flow and confound measurements.<sup>11</sup> Finally, many agents, especially endothelium dependent vasodilators (for example, acetylcholine, see fig 1), have a near instantaneous onset and offset of action, and performing QCA a minute after injection<sup>12</sup> is likely to result in misleading measurements.

The use of IVUS can circumvent many of these limitations and provide continuous ECG gated cross sectional area and compliance measurements of the coronary artery. In addition, IVUS provides more detailed morphometric information of the coronary artery including plaque volume, composition, severity, and distribution. Moreover, the combined use of IVUS and Doppler wire does facilitate the functional assessment of both conduit and resistance vessel function.<sup>13</sup> Although cross talk can occur between the IVUS and Doppler systems owing to frequency overlap, meaningful image or signal loss is unusual and rarely affects cross sectional area or velocity measurements. However, the IVUS imaging catheter (cross

Table 1 Intracoronary administration of agents commonly used in the functional assessment of the coronary circulation

Agent	Dose	Receptor/mediator	Comments
<i>Endothelium independent vasodilators</i>			
Adenosine	Bolus: 12–36 µg Infusion: 1–2 mg/min	Purine receptors	Causes transient heart block. Causes maximal coronary vasodilatation and is also used for the assessment of fractional flow reserve
Glyceryl trinitrate	Bolus: 50–200 µg	Nitric oxide donor	Predominant action on epicardial vessels
Papaverine	Bolus: 4–12 mg	Opiate derivative causing vascular smooth muscle relaxation	Causes maximal coronary vasodilatation. Potentially arrhythmogenic
Sodium nitroprusside	Infusion: 5–40 µg/min	Nitric oxide donor	Predominant action on coronary resistance vessels
<i>Endothelium dependent vasodilators</i>			
Acetylcholine	Bolus: 1–100 nmol Infusion: 1–1000 nmol/min	Muscarinic receptors	Causes transient heart block. Target effective intracoronary concentration of 10 <sup>-6</sup> to 10 <sup>-4</sup> M. May cause paradoxical vasoconstriction in presence of atheroma
Bradykinin	Bolus: 60–600 pmol Infusion: 30–2500 pmol/min	Bradykinin type 2 receptor	Tachyphylaxis and chest discomfort may occur
Substance P	Infusion: 5–40 pmol/min	Neurokinin type 1 receptor	
<i>Endothelium dependent vasoconstrictors</i>			
L-NMMA	Infusion: 32–64 µmol/min	Nitric oxide synthase inhibitor	Slow onset and offset of action (10–20 minutes)

Boluses should be given in 2 ml followed by 3 ml saline flush. The bolus dose should be reduced by 30–50% for right coronary artery injection.

To avoid directly influencing coronary flow, infusion rates are usually low at 1–2 ml/min.

Adenosine and papaverine may additionally cause endothelium dependent flow associated epicardial vessel vasodilatation.

Except L-NMMA, all agents have a rapid onset and offset of action with flow velocity usually returning to baseline within ~2 minutes.

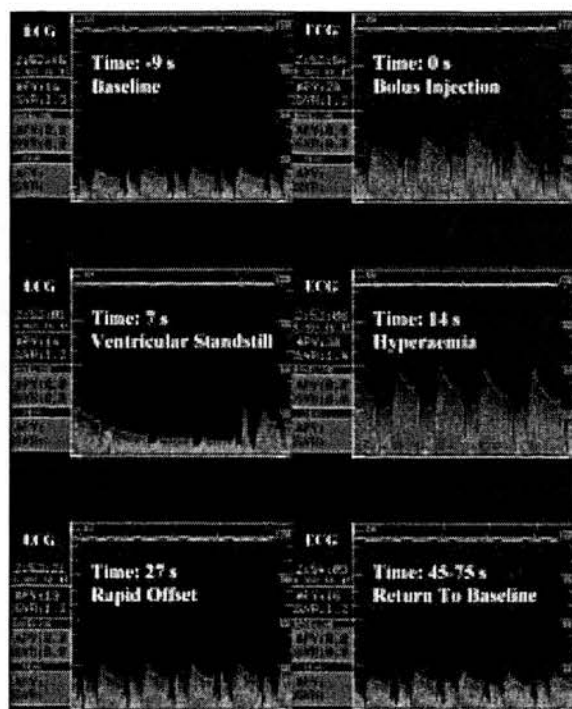


Figure 1 Doppler flow velocity responses before, during, and following a bolus injection of acetylcholine (2 ml of  $10^{-4}$  M) in the dominant circumflex coronary artery.

sectional area  $\sim 0.9$  mm<sup>2</sup>) can cause significant obstruction to flow, particularly in the presence of luminal stenoses of  $\geq 70\%$  and, therefore, heavily diseased arterial segments or small calibre coronary arteries are not suited to this approach and limit its more widespread application. Moreover, unlike QCA, IVUS provides a single cross sectional image of the artery at a given time point, and although three dimensional reconstructions of the artery can be performed, it does not permit an instantaneous assessment of the entire arterial tree.

#### Method of drug administration

The method of administration of vasoactive agents under investigation has been variable and inconsistent. For example, acetylcholine has been administered as a continuous infusion,<sup>10-14</sup> slow hand injection<sup>15</sup> or rapid bolus.<sup>12</sup> Moreover, instillation of drug into the coronary circulation has been achieved either via the instrumented catheter<sup>14-16</sup> or a dedicated 2-3 French selective intracoronary infusion catheter.<sup>17-18</sup> Indeed, some workers have used selective monorail infusion catheters which have remained within the guide catheter.<sup>19</sup> Finally, systemic intravenous infusions of agents, such as adenosine (140  $\mu$ g/kg/min), have been administered but this approach can cause pronounced systemic haemodynamic and arrhythmogenic effects which will confound the subsequent interpretation of coronary responses.<sup>20</sup>

#### BOLUS INJECTIONS

Consideration should be given to the catheter dead space which can be significant, particularly when drugs are given via the guide catheter. The administration of even a small volume bolus will necessitate a 3 ml saline flush to eject the drug from the catheter into the proximal coronary artery. This will cause an instantaneous increase in blood flow velocity which is attributable to the mechanical ejection of fluid down the artery. Thereafter, a second rise or a subse-

quent fall in blood flow velocity will occur which is attributable to drug action (fig 1). Prolonged injection or large volume boluses have the potential to obscure the second phase response because of superimposition of mechanical and pharmacological flow effects as well as inducing shear stress and flow associated dilatation. Bolus injections should, therefore, be kept to a minimal volume and must be compared with control saline injections. Finally, bolus injections of acetylcholine and adenosine into the right coronary or dominant circumflex artery can result in atrio-ventricular block and transient ventricular standstill (fig 1). If prolonged, this will confound the assessment of vasodilatation and flow responses, and continuous infusions of acetylcholine or adenosine into these arteries should be avoided.

#### CONTINUOUS OR GRADED INFUSIONS

The administration of drugs via the diagnostic or guide catheter may be satisfactory for the application of drug boluses by hand injection when maximal vasodilatory responses are being assessed, such as with high dose (30  $\mu$ g bolus) adenosine. However, there is a concern that continuous or graded infusions via the coronary guide catheter do not reliably permit precise and selective intracoronary drug administration. The turbulence induced by blood ejection from the heart and the potential incomplete engagement of the catheter with the coronary ostium will result in a variable degree of drug reflux into the aorta. Furthermore, in the left coronary system, a variable amount of the delivered drug will be administered to the adjacent epicardial vessel. Consequently, there is the theoretical concern that guide catheter infusion will result in a wide variability in the effective intracoronary drug concentration attained. When assessing the coronary vasomotor response by QCA, these concerns are further compounded by the necessity to aspirate the drug from the diagnostic or guide catheter before contrast injection.<sup>10-14</sup> Finally, impaction of the guide catheter in the coronary ostium due to superselection, or the use of large guide catheters, should clearly be avoided as this will impair antegrade coronary flow. Administration of drugs through guide catheters with side holes is equally inappropriate, and it is likely that smaller guide catheters will increasingly be used with the wider use of 6 French compatible IVUS catheters.

We have recently been conducting a study to look at the relation between endothelial function and atheromatous plaque volume in the coronary circulation of patients with normal or mildly diseased coronary arteries. The plaque volume of the proximal left anterior descending coronary artery was determined using three dimensional reconstruction of an initial IVUS examination, and coronary blood flow responses to incremental five minute infusions (1 ml/min) of substance P were assessed by combined Doppler wire and IVUS measurements. In 10 patients, substance P was selectively infused via the flush port of the IVUS catheter which caused significant and consistent increases in coronary blood flow (fig 2). However, in a further 10 patients, where substance P was administered via the guide catheter, the magnitude and consistency of the coronary blood flow response was low and did not result in significant increases in coronary blood flow despite a comparable degree of proximal coronary atheroma (6.2 (1.0) v 5.8 (1.4) mm<sup>2</sup>/mm of vessel, respectively). Therefore, we believe that, to achieve reproducible vasomotor responses, continuous or graded intracoronary drug administration should be given using a selective intracoronary infusion catheter which, to date, has not been universally employed.



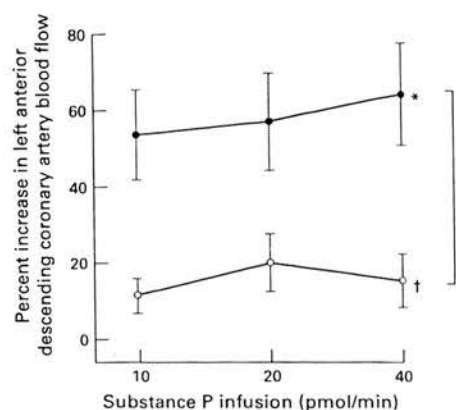


Figure 2 Coronary blood flow response to incremental substance P infusion (1 ml/min) administered via the guide catheter (open circles) or IVUS catheter (closed circles).  $n = 10$  per group. One way analysis of variance (ANOVA) \* $p < 0.001$ ; † $p$  = not significant. Two way ANOVA \* $p < 0.001$ .

### Conclusions

The measurement of coronary blood flow responses to vasoactive agents and investigational agents is an important and essential area of clinical research. However, it would appear that the method of assessment and the route of intracoronary drug administration will have a significant influence on subsequent coronary vasomotor responses. Consequently, the method and technique used will depend on the specific question under investigation and should be guided by the limitations of each approach.

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**PUBLICATION 33**

## Blood Coagulation, Fibrinolysis and Cellular Haemostasis

# Local tissue factor pathway inhibitor release in the human forearm

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### Summary

Nineteen healthy men received unilateral brachial artery infusions of either unfractionated heparin (0.3–100 IU/min), saline or the endothelium-dependent vasodilators substance P (2–8 pmol/min) and bradykinin (100–1000 pmol/min), and the endothelium-independent vasodilator sodium nitroprusside (2–8 µg/min). Heparin caused a dose-dependent increase in plasma TFPI concentrations in both arms (ANOVA,  $p < 0.0001$ ). Estimated net forearm TFPI release was  $7 \pm 16$ ,  $29 \pm 20$  and  $138 \pm 72$  ng/100 mL tissue/min during 10, 30 and 100 IU/min of

heparin respectively (ANOVA,  $p < 0.0001$ ). Compared to the systemic circulation, the forearm sensitivity to heparin induced TFPI release was 3.6-fold lower ( $166 \pm 67$  ng/IU vs.  $596 \pm 252$  ng/IU; t-test,  $p = 0.004$ ). Substance P, bradykinin and sodium nitroprusside all caused substantial dose-dependent increases in blood flow (ANOVA,  $p < 0.001$  for all) without affecting plasma TFPI concentrations. There are important regional differences in endothelial TFPI release, with the forearm circulation being relatively insensitive to heparin.

### Keywords

Tissue factor pathway inhibitor, haemostasis, heparin, substance P, bradykinin

Thromb Haemost 2003; 89: 438–45

## Introduction

Tissue factor pathway inhibitor (TFPI) is a Kunitz-type protease inhibitor that regulates the extrinsic coagulation cascade (1). It is synthesised and released by endothelial cells, where it is expressed on the cell surface as well as stored within intracellular secretory granules (2). In addition to inhibiting tissue factor-factor VIIa complex, TFPI directly neutralises the catalytic activity of factor Xa. The plasma form of TFPI is predominantly present as a truncated C-terminal fragment that is a less efficient inhibitor of tissue factor and represents only a small fraction of the available TFPI (3). As such, changes in TFPI activity are likely to be dependent on endothelial function, and in particular on the acute cellular release and expression of TFPI.

An imbalance between TFPI and tissue factor concentrations may be important in the pathogenesis of many conditions

associated with elevated tissue factor expression, such as atherosclerosis (4–6), restenosis after angioplasty (7), sepsis (8), diabetes mellitus (9), adult respiratory distress syndrome (10) and glomerulonephritis (11). Such an imbalance may arise because of impaired endothelial function (12) and lead to a procoagulant state (13, 14). Administration of exogenous (recombinant) TFPI may be beneficial in many of these clinical situations (8, 13, 15–17) although the potential role of increasing endogenous TFPI availability has yet to be tested. Understanding the control of endogenous TFPI release may thus provide novel therapeutic opportunities in the treatment of tissue factor-mediated pathological conditions.

To date, few stimuli have been reported to induce acute TFPI release apart from shear stress, heparin and other negatively charged ions (18–21). The *in vivo* assessment of TFPI release in response to alterations in haemodynamics and shear stress

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presents several challenges. First, the liver is responsible for rapid clearance of TFPI from the circulation and changes in hepatic blood flow can have considerable effects on plasma TFPI concentrations (22). Second, systemic drug administration can cause concomitant effects on other organ systems, such as the brain, kidney and heart, as well as influence neurohumoral reflexes. Therefore, alterations of systemic as well as hepatic haemodynamic variables could confound any assessment of acute TFPI release. In contrast, the use of bilateral forearm blood flow measurements coupled with unilateral brachial artery infusion of vasoactive drugs at subsystemic, locally active doses, provides a powerful and reproducible method of directly assessing vascular responses *in vivo* (23). Combined with bilateral forearm venous sampling, this technique permits the assessment of local release of tissue and endothelium-derived factors (24). However, to date, this model has not been applied to the assessment of acute endothelial TFPI release *in vivo* in man. We, therefore, chose to assess the acute local forearm release of TFPI in response to intra-arterial heparin infusion since heparin has been previously demonstrated to be one of the most potent compounds to induce its release (25). Using this model, we also wished to determine whether endothelium-dependent or -independent vasodilators would cause local TFPI release in the human forearm circulation.

## Methods

### Subjects

Nineteen healthy non-smoking male volunteers subjects (age 19-35 years) participated in the study which was undertaken with the approval of the local research ethics committee, in accordance with the Declaration of Helsinki, and with the written informed consent of each subject. None of the subjects received vasoactive drugs in the week before each phase of the study. All abstained from caffeine-containing drinks and alcohol for at least 12 h before each study. All studies were carried out in a quiet, temperature controlled room maintained at 22-24° C.

### Study design

The brachial artery of the non-dominant arm was cannulated with a 27-standard wire gauge steel needle (Cooper's Needle Works Ltd, Birmingham, UK) under local anaesthesia (1% lignocaine; Astra Pharmaceutical Ltd, Kings Langley, UK). The total rate of intra-arterial infusions was maintained constant at 1 mL/min and forearm blood flow was measured every 10 min throughout all studies. Saline was infused for 30 min prior to the heparin, saline, substance P, sodium nitroprusside and bradykinin infusions.

### Protocol 1

Unfractionated heparin was obtained from Akzo Nobel (Oss, The Netherlands) and prepared for administration by the

pharmacy of the Leiden University Medical Center (Leiden, The Netherlands) following European Pharmacopoeia standards. In 6 subjects, heparin was infused at escalating doses of 0.3, 1.0, 3.0, 10, 30, and 100 IU/min for 10 min at each dose. For safety purposes, the activated partial thromboplastin time (Coagucheck Plus, Roche Diagnostics, Mannheim, Germany) was measured in the non-infused arm during the 10, 30 and 100 IU/min heparin infusions. Six additional subjects also underwent a control 2 h intra-arterial saline infusion.

### Protocol 2

This was performed as a sub-study (placebo day) of a previously reported clinical investigation (26) in seven subjects. Intra-brachial infusions of substance P (Clinalfa AG, Läufelfingen, Switzerland; endothelium-dependent vasodilator) at 2, 4 and 8 pmol/min (24), sodium nitroprusside (David Bull Laboratories, Warwick, UK; endothelium-independent vasodilator) at 2, 4 and 8 µg/min (24, 27) and bradykinin (Clinalfa AG; endothelium-dependent vasodilator) at 100, 300 and 1000 pmol/min (27) were given for 10 min at each dose in that order. Saline was infused for 30 min prior to the substance P, sodium nitroprusside and bradykinin infusions.

## Measurements

### Forearm blood flow and haemodynamics

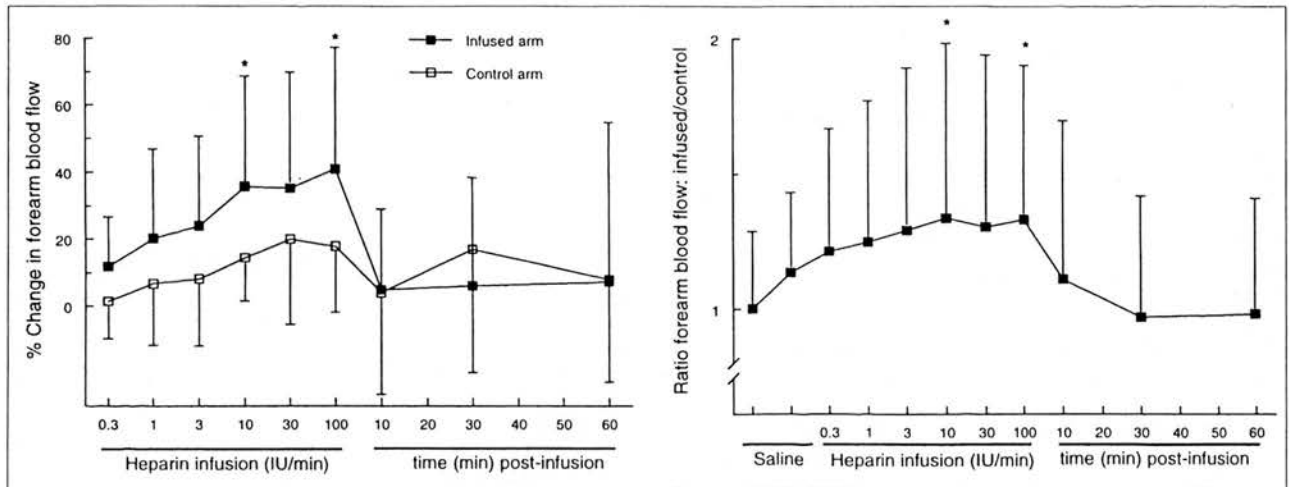
Blood flow was measured in both forearms by venous occlusion plethysmography using mercury-in-silastic strain gauges applied to the widest part of the forearm as previously described (23). Analogue voltage output from an EC-4 Strain Gauge Plethysmograph (D. E. Hokanson Inc, Washington, USA) was processed by a MacLab<sup>®</sup> analogue-to-digital converter and Chart<sup>™</sup> v3.3.8 software (AD Instruments Ltd, Castle Hill, Australia) and recorded onto a Macintosh Classic II computer (Apple Computers Inc, Cupertino, USA). Calibration was achieved using the internal standard of the plethysmograph.

Blood pressure and heart rate were monitored in the non-infused arm at intervals throughout each study using a semi-automated non-invasive oscillometric sphygmomanometer (Takeda UA 751, Takeda Medical Inc, Tokyo, Japan).

### Blood sampling and assays

Venous cannulae (17G) were inserted into large subcutaneous veins of the antecubital fossa in both arms. Ten to twenty mL of blood was withdrawn simultaneously from each arm and collected into citrate tubes (Monovette<sup>®</sup>, Sarstedt, Nümbrecht, Germany), and kept on ice before being centrifuged at 2000 g for 30 min at 4° C. Platelet poor plasma was decanted, snap-frozen in methanol with dry-ice, and stored at -80° C until analysis.

Plasma total and free TFPI concentrations were determined using enzyme-linked immunosorbent assays (Asserachrom<sup>®</sup>,



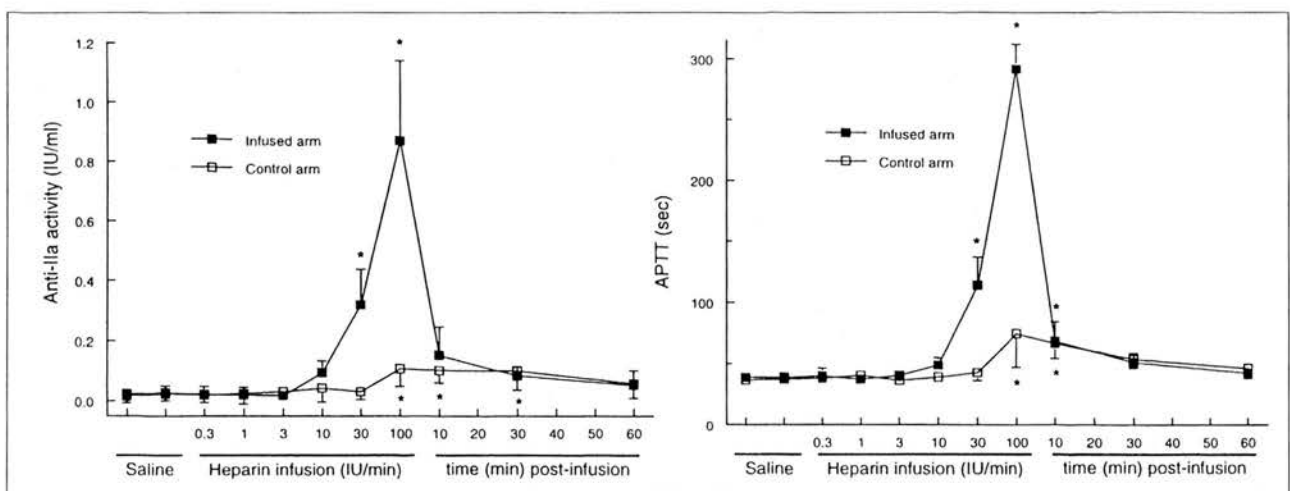
**Figure 1:** Percent change in forearm blood flow (compared to baseline) and ratio infused/control arm  $\pm$  SD during intra-arterial heparin infusion (10 minutes each dose) and post-infusion. ANOVA  $p = 0.066$  for the infused arm percent change and  $p = 0.024$  for the ratio, \*  $p < 0.05$  compared to baseline (Dunnett's)

Diagnostica Stago, Asnières, France). The range, and the intra- and inter-assay coefficients of variation of the plasma total and free TFPI concentrations were 4-230 ng/mL, 4.2% and 6.8%, and 2-70 ng/mL, 3.9% and 4.4% respectively. The anti-IIa activity assay (Spectrolyse<sup>®</sup> Heparin) was calibrated against the first International Standard from the National Institute for Biological Standards and Control (Hertsfordshire, United Kingdom; code 85/600) with intra- and inter-assay coefficients of variation of  $<10\%$  and a sensitivity of 0.01 IU/ml. The activated partial thromboplastin time (APTT) was determined on a STA<sup>®</sup> coagulation analyser (Roche Diagnostics, Mannheim, Germany) using STA APTT reagent (Roche Diagnostics) with intra- and inter-assay coefficients of variation

of  $<5\%$ . Plasma t-PA and PAI-1 antigen and activity concentrations were determined using enzyme-linked immunosorbent assays and a photometric method as previously described (24).

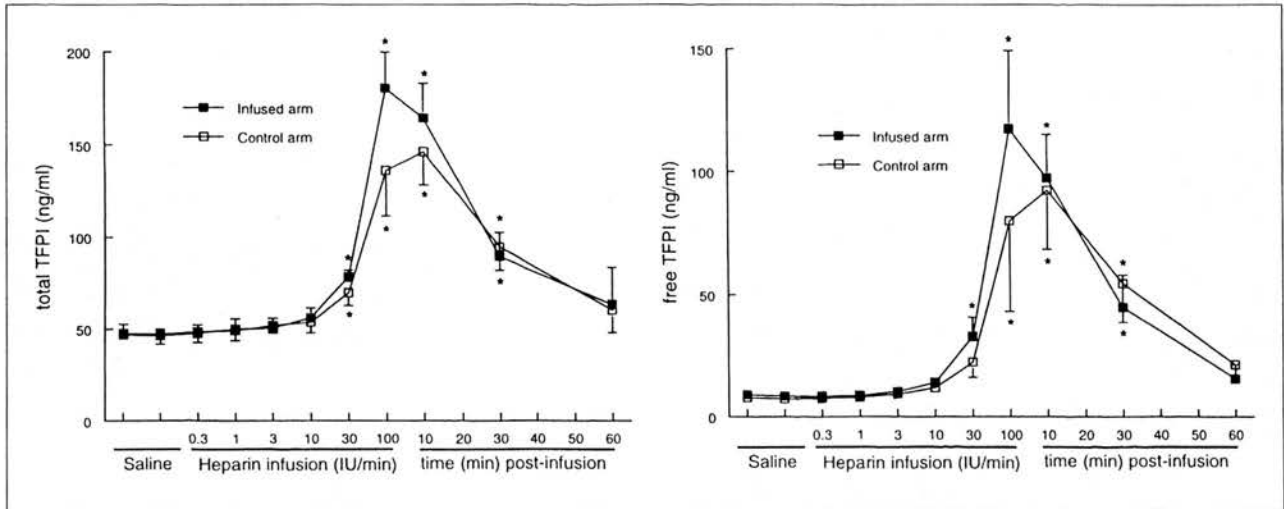
### Data analysis and statistics

Plethysmographic data were extracted from the Chart<sup>™</sup> data files. Forearm blood flows were calculated for individual venous occlusion cuff inflations by use of a template spreadsheet (Excel v5.0; Microsoft Corporation, Cambridge, USA) as previously described (24, 27). Estimated net release of total and free TFPI was defined as the product of the infused forearm



**Figure 2:** Anti-IIa activity and APTT  $\pm$  SD pre-dose (saline), during intra-arterial heparin infusion (10 min each dose) and post-infusion. ANOVA  $p < 0.0001$  for both arms, \*  $p < 0.05$  compared to baseline (Dunnett's)





**Figure 3:** Total and free tissue factor pathway inhibitor (TFPI) plasma concentration  $\pm$  SD during pre-dose (saline), intra-arterial heparin infusion (10 min each dose) and post-infusion. ANOVA  $p < 0.0001$  for both arms, \*  $p < 0.05$  compared to baseline (Dunnett's)

plasma flow (based on the mean haematocrit and the infused forearm blood flow) and the concentration difference between the infused and non-infused arms (24). The anti-IIa activity was used as a measure of heparin concentration (28) to assess concentration-effect relationships. Average concentration-effect relationships were calculated from the individual regression lines.

Statistical analysis was performed, where appropriate, by repeated measures analysis of variance (ANOVA), two-tailed Dunnett's test, paired Student's *t*-tests and regression analysis using SAS for Windows V8.2 (SAS Institute Inc., Cary, NC, USA). Statistical significance was taken as  $p < 0.05$ .

## Results

All studies were well tolerated without significant adverse effects. Consistent with previous studies (27), transient patchy flushing and mild skin oedema of the infused arm occurred with bradykinin and substance P infusion.

### Protocol I

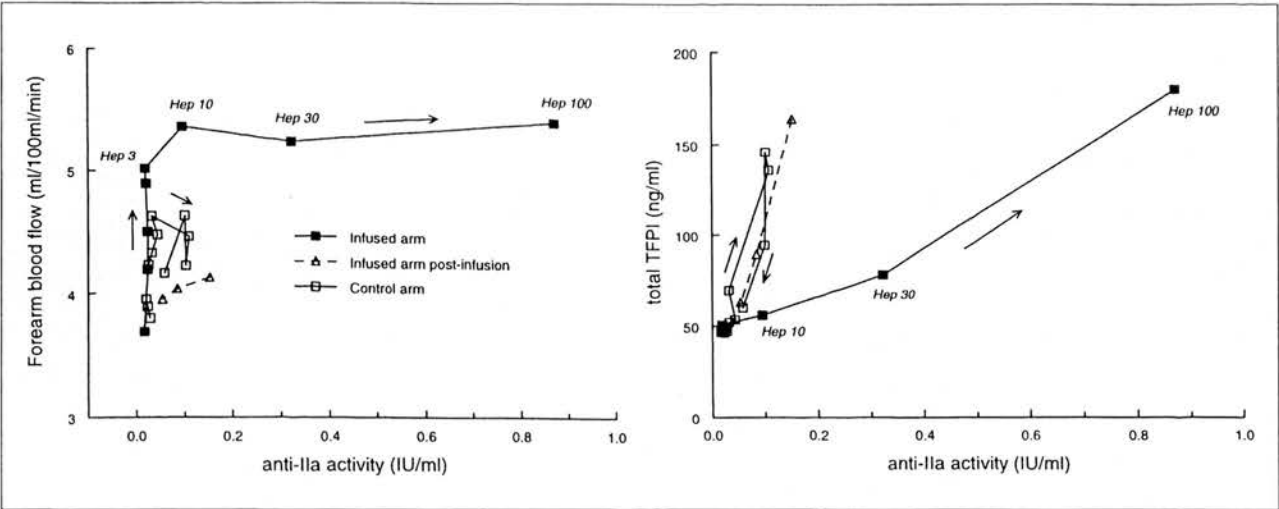
#### Vasomotor effects

Saline infusion had no effect on forearm blood flow in either arm (data not shown). There was a trend (ANOVA,  $p = 0.066$ ) for heparin infusion to cause an increase in blood flow of the

**Table 1:** Release of tissue factor pathway inhibitor (TFPI) with heparin infusion.

Time (min)	Heparin (IU/min)	Concentration Difference Between Forearms <sup>†</sup> (ng/mL)	Estimated Net Release (ng/100 mL/min)
-10	0	$-0.6 \pm 3.9$	$-2.1 \pm 7.0$
0	0	$-0.9 \pm 3.2$	$-0.5 \pm 9.0$
10	0.3	$-0.8 \pm 2.4$	$-0.6 \pm 7.7$
20	1	$0.7 \pm 4.0$	$1.3 \pm 11.0$
30	3	$-1.3 \pm 3.5$	$-2.8 \pm 11.2$
40	10	$2.5 \pm 5.0$	$7.0 \pm 15.7$
50	30	$8.6 \pm 4.9$	$28.8 \pm 20.4$
60	100	$44.4 \pm 18.6^*$	$138.1 \pm 71.7^*$
70	0	$18.3 \pm 17.6^*$	$40.7 \pm 38.6$
90	0	$-5.3 \pm 5.8$	$-10.2 \pm 16.8$
120	0	$3.1 \pm 11.3$	$16.0 \pm 37.5$

<sup>†</sup>  $p < 0.0001$  ANOVA with factor time  
\*  $p < 0.05$  compared to mean baseline, Dunnett's correction for multiple comparison



**Figure 4:** Relationship between anti-IIa activity (measure for heparin concentration) and forearm blood flow (*left panel*) and total tissue factor pathway inhibitor (TFPI) plasma concentration (*right panel*). Arrows indicate direction of dose response. Relationship between anti-IIa activity and total TFPI was 3-4 fold weaker ( $p = 0.0035$ ) in the infused arm compared to the control arm.

infused arm (maximum average increase  $41 \pm 36\%$ ; 95% confidence interval, 5-77%: Fig. 1) that appeared to be dose-dependent. The ratio of the infused arm and non-infused arm blood flow increased in a dose-dependent manner (ANOVA,  $p = 0.024$ : Fig. 1).

**Anticoagulant effects and TFPI release**

Isolated saline infusion had no effect on plasma total TFPI concentrations in either arm at 0, 60 and 120 min (data not shown). Heparin induced a dose-dependent increase in anti-IIa activity and APTT at >10 IU/min in the infused arm (ANOVA,  $p < 0.0001$  for both) and at 100 IU/min in the non-infused arm (ANOVA,  $p < 0.0001$  for both; Fig. 2). Anti-IIa activity and

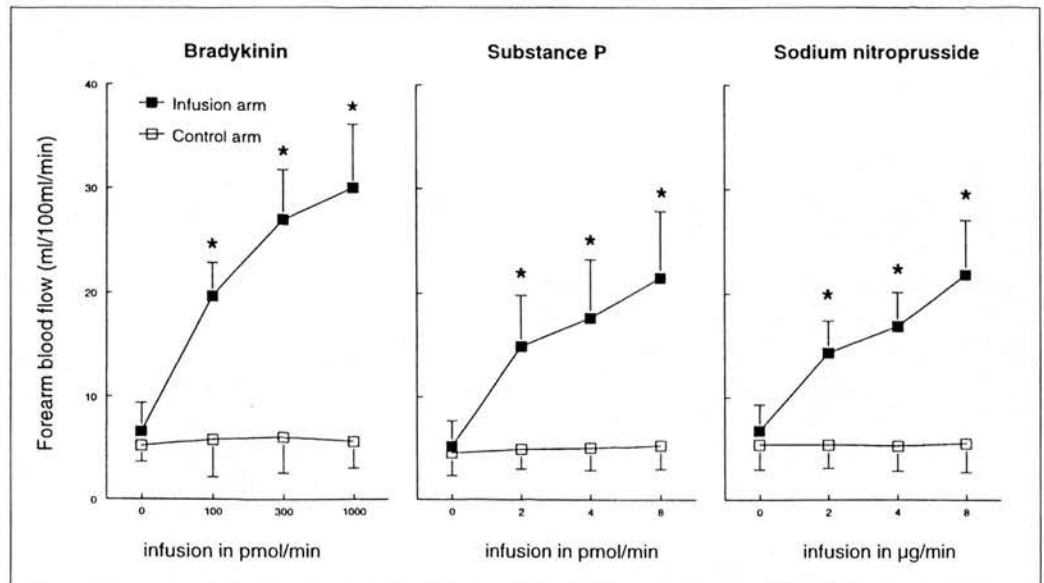
APTT returned to baseline values 60 min after cessation of the heparin infusion.

Plasma total and free TFPI concentrations increased dose-dependently in both the infused and non-infused arms (ANOVA,  $p < 0.0001$  for all; Fig. 3). At lower heparin doses, plasma TFPI concentrations appeared to increase before the anti-IIa activity (Fig. 2 and 3). Plasma TFPI concentrations were higher in the infused arm with a dose-dependent increase in the forearm difference of the total TFPI concentration at 30 and 100 IU/min of heparin (ANOVA,  $p < 0.0001$ : Table 1). Plasma total and free TFPI concentrations were similar in both arms after cessation of the heparin infusion. The estimated net forearm release of total TFPI increased to  $7 \pm 16$ ,  $29 \pm 20$  and

Drug	Dose	Concentration Difference Between Forearms (ng/ml)	Estimated Net Release (ng/100 mL/min)
Bradykinin (pmol/min)	0	$-2.7 \pm 4.1$	$-11.7 \pm 15.5$
	100	$-3.0 \pm 5.1$	$-35.4 \pm 69.2$
	300	$-2.4 \pm 6.9$	$-51.1 \pm 118.7$
	1000	$-5.7 \pm 7.4$	$-110.4 \pm 148.1$
Substance P (pmol/min)	0	$1.4 \pm 5.1$	$0.9 \pm 21.8$
	2	$-0.4 \pm 3.6$	$-6.4 \pm 40.8$
	4	$-1.5 \pm 4.8$	$-16.1 \pm 46.6$
	8	$-7.3 \pm 4.3$	$-96.9 \pm 74.6$
Sodium Nitroprusside ( $\mu$ g/min)	0	$-1.2 \pm 2.9$	$-6.2 \pm 11.9$
	2	$1.3 \pm 4.9$	$8.2 \pm 42.0$
	4	$-3.1 \pm 4.2$	$-40.2 \pm 47.8$
	8	$3.6 \pm 6.3$	$41.3 \pm 79.7$

**Table 2:** Release of tissue factor pathway inhibitor (TFPI) with bradykinin, substance P and sodium nitroprusside infusion.

**Figure 5:** Forearm blood flow in response to intra-brachial infusion of bradykinin, substance P and sodium nitroprusside (ANOVA,  $p < 0.001$  for all).



$138 \pm 72$  ng/100 mL of tissue/min with 10, 30 and 100 IU/min of heparin respectively (ANOVA,  $p < 0.0001$ : Table 1).

#### Concentration-effect relationship

Forearm blood flow increased with anti-IIa activity but appeared to reach a maximum effect at 10 IU/min of heparin (Fig. 4).

With increasing anti-IIa activity, the proportionate rises in plasma total and free TFPI concentrations were smaller in the infused arm (total TFPI  $166 \pm 67$  ng/IU, free TFPI  $137 \pm 63$  ng/IU) than in the non-infused arm (total TFPI  $596 \pm 252$  ng/IU, free TFPI  $532 \pm 196$  ng/IU;  $p = 0.0035$ : Fig. 4).

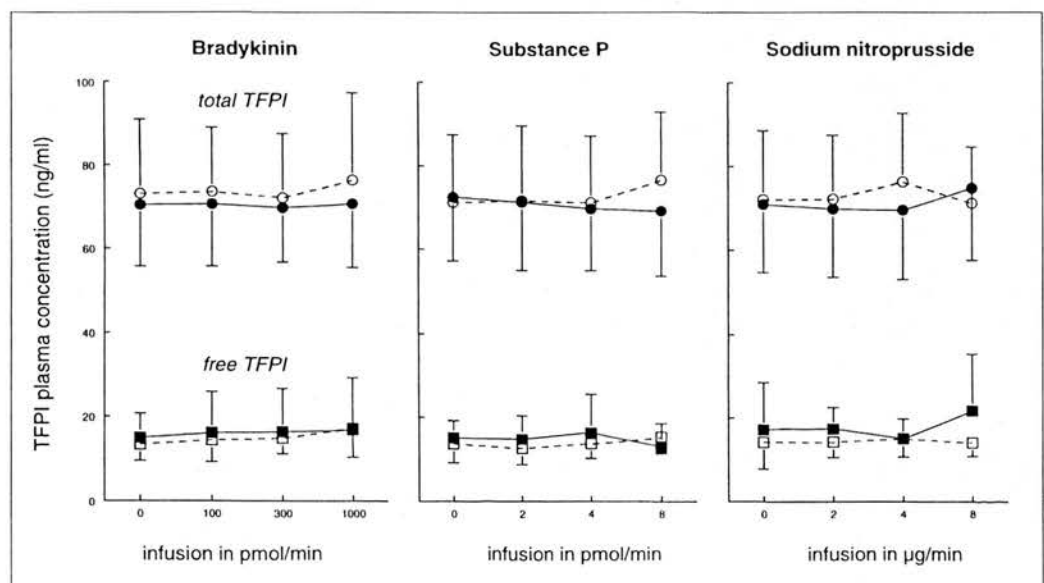
The estimated net TFPI release in the infused arm directly correlated with the anti-IIa activity (average relation  $y = -6 + 159x$ , 95% confidence interval  $-18$  to  $7$  for the intercept and  $82$  to  $236$  for the slope;  $r = 0.94$ - $0.98$  for the individual regression lines,  $p < 0.01$ ).

#### Protocol 2

##### Vasomotor effects

Substance P, bradykinin and sodium nitroprusside produced a dose-dependent increase in blood flow (ANOVA,  $p < 0.001$  for all; Fig. 5). There were no significant changes in heart rate, blood pressure or blood flow in the non-infused forearm.

**Figure 6:** Plasma concentrations of total TFPI (circles) and free TFPI (squares) in the infusion arm (closed markers) and control arm (open markers) in response to intra-brachial infusion of bradykinin, substance P and sodium nitroprusside.



**Table 3:** Plasma tissue plasminogen activator (t-PA) antigen and activity concentrations in the infused and non-infused forearms during substance P, sodium nitroprusside and bradykinin infusion (Protocol 2) (25).

	Substance P (pmol/min)				Sodium Nitroprusside (µg/min)				Bradykinin (pmol/min)				
	0	2	4	8	0	2	4	8	0	100	300	1000	
Plasma t-PA Antigen (ng/mL)													
Infused Arm	3.9 ± 0.8	4.8 ± 0.8	5.8 ± 0.7	7.5 ± 1.1	† 3.3 ± 0.4	3.1 ± 0.5	2.8 ± 0.7	3.8 ± 0.7	3.6 ± 0.6	5.1 ± 0.9	8.0 ± 2.0	13.2 ± 4.4	†
Non-infused Arm	4.0 ± 0.9	5.3 ± 1.5	4.1 ± 0.8	4.6 ± 1.0	3.9 ± 0.8	3.7 ± 0.8	3.8 ± 0.7	3.4 ± 0.6	3.3 ± 0.5	3.8 ± 1.2	4.2 ± 1.1	6.0 ± 2.3	*
Plasma t-PA Activity (IU/mL)													
Infused Arm	1.8 ± 0.2	2.5 ± 0.4	3.7 ± 0.9	4.8 ± 1.1	† 1.9 ± 0.4	1.5 ± 0.1	1.5 ± 0.1	1.6 ± 0.2	1.5 ± 0.2	2.5 ± 0.3	4.5 ± 0.9	8.5 ± 2.0	†
Non-infused Arm	1.9 ± 0.2	2.0 ± 0.3	1.8 ± 0.2	1.8 ± 0.2	1.8 ± 0.2	1.9 ± 0.3	1.7 ± 0.2	1.7 ± 0.2	1.7 ± 0.2	1.8 ± 0.2	2.2 ± 0.2	2.4 ± 0.4	*
†p<0.001, *p<0.05 ANOVA (dose response)													

**Fibrinolytic effects and TFPI release**

Substance P and bradykinin, but not sodium nitroprusside, caused a dose-dependent increase in plasma t-PA concentrations (ANOVA, p <0.001 for all; Table 2). There were no changes in plasma total and free TFPI or PAI-1 concentrations during substance P, bradykinin, or SNP infusion (Fig. 6 and Table 4). There were no changes in the concentration differences between the forearms or the estimated net release of TFPI in the infused arm (Table 2).

**Discussion**

We have shown that intra-arterial heparin causes modest dose-dependent increases in local TFPI release directly from the forearm vascular bed. This effect is specific to heparin since neither marked vasodilatation induced by sodium nitroprusside nor endothelial cell stimulation with the kinins, bradykinin and substance P, were able to release TFPI despite marked increases in blood flow and tissue plasminogen activator respectively. However, in comparison to the systemic vasculature, the forearm appears to have a relatively low sensitivity for heparin induced TFPI release.

Intra-arterial heparin caused a dose-dependent increase in TFPI release in the forearm, but this was associated with near simultaneous increases in systemic plasma TFPI concentrations. This cannot be attributable to an “overspill” of TFPI into the

systemic circulation since insufficient TFPI was released from the forearm and the plasma half-life for TFPI is too brief due to its rapid clearance by the liver (~700 mL/min) (22). At 30 IU/min, heparin caused the forearm release of 0.3 µg/min of TFPI whereas, in the absence of any clearance, the forearm would need to produce at least 13 µg/min of TFPI to raise systemic plasma concentrations to 20 ng/mL.

The concentration-effect relationship of intra-arterial heparin (anti-IIa activity) and systemic plasma TFPI concentrations was similar to our previous observations with intravenous heparin administration (28). However, this relationship was 3-4 fold weaker in the forearm circulation: 0.1 IU anti-IIa activity causing a 17 ng/mL increase in the forearm compared with a 60 ng/mL increase in the systemic circulation. The difference between local and systemic TFPI release will be underestimated by such comparisons since the increase in forearm concentration will be in part the result of systemically released TFPI. Moreover, the systemic concentration-effect relationship is probably steeper since TFPI is rapidly cleared by the liver (22). These inferred differences in release rates are most likely due to regional vascular differences in endothelial TFPI release. In the present study, we cannot determine which vascular bed is responsible for the major source of heparin induced TFPI release but the skeletal muscle vasculature does not appear to have a major role. Whichever vascular bed is responsible, it is

	Substance P (pmol/min)		Sodium Nitroprusside (µg/min)		Bradykinin (pmol/min)	
	0	8	0	8	0	1000
<b>Plasma PAI-1 Antigen (ng/mL)</b>						
Infused Arm	14 ± 2	14 ± 1	14 ± 3	14 ± 2	16 ± 2	11 ± 1
Non-infused Arm	15 ± 4	15 ± 1	12 ± 2	14 ± 2	10 ± 1	13 ± 2

**Table 4:** Plasma plasminogen activator inhibitor type I (PAI-I) antigen concentrations in the infused and non-infused forearms during substance P, sodium nitroprusside and bradykinin infusion (Protocol 2).

very sensitive to the effects of heparin since the rise in systemic plasma TFPI concentrations preceded the increase in anti-IIa activity. This also reflects the relative insensitivity of the anti-IIa activity assay as a biomarker for active heparin plasma concentrations.

The endothelium-dependent vasodilators substance P (24), bradykinin (27) and the  $\beta$ -receptor agonist isoproterenol (29) increase blood flow and endothelial tissue plasminogen activator (t-PA) release. Since both TFPI and t-PA are stored within intracellular vesicles and their release is dependent on intracellular calcium ions (30), we hypothesised that endothelial stimulants or increases in blood flow would also lead to TFPI release. We here report that, despite substantial increases in blood flow and t-PA release, we were unable to detect increases in plasma TFPI

concentrations using either endothelium-dependent or -independent vasodilators. Given that heparin also does not release t-PA (31), it would appear that the pathways of t-PA and TFPI release are mediated through distinct cellular mechanisms. This is not entirely unexpected given that previous work has shown release of other endothelium-derived factors, such as von Willebrand factor, are also not induced by substance P (24) or bradykinin (27).

In conclusion, although neither vasodilatation nor direct kinin-mediated endothelial cell stimulation have an effect, unfractionated heparin causes a modest direct local release of TFPI in the human forearm. There are important regional differences in endothelial TFPI release with the forearm circulation being relatively insensitive to heparin.

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**PUBLICATION 34**

## Endothelial fibrinolytic function in hypertension: the expanding story

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See original paper on page 1551

In recent years, impairment of endothelial function has been a major focus of clinical research into cardiovascular disease. Endothelial dysfunction is associated with classical risk factors and there is accumulating evidence that it also independently predicts cardiovascular outcome [1]. This research has almost exclusively focused on endothelium-dependent vasodilatation as a measure of endothelial function. However, although this is important, it may not be representative of other important aspects of endothelial function, such as the regulation of fibrinolysis.

Following the initiation of intravascular thrombus formation, the endothelium acutely releases tissue-type plasminogen activator (t-PA) in response to a range of factors predominantly related to the coagulation cascade, especially factor Xa and thrombin [2]. Once released, t-PA catalyses the conversion of plasminogen to plasmin and thereby facilitates thrombus dissolution through the proteolytic degradation of fibrin to soluble fibrin degradation products. This endogenous fibrinolytic system protects the circulation from intravascular fibrin formation and thrombosis that would otherwise result in vessel occlusion and tissue ischaemia. It follows that impairment of acute t-PA release from the endothelium might be a specific mechanism through which endothelial dysfunction mediates or potentiates thrombotic events.

Jern and colleagues [3] have previously reported that desmopressin-induced endothelial t-PA release is significantly impaired in patients with hypertension and have since explored the mechanism responsible for this observation. Using an *ex vivo* perfusion model in which shear stress was kept constant, increased intraluminal pressure in human umbilical veins decreased t-PA release, as well as gene and protein expression. This suggested that raised intraluminal pressure *per se* may be important in mediating impaired t-PA release in hypertension. The

authors have now extended these findings and have specifically investigated the effects of cyclic tensile, rather than compressive, strain on endothelial t-PA production and release. In this issue of the journal, Ulfhammer *et al.* [4] show that cyclically stretching cultured human aortic endothelial cells (HAECs) decreases both t-PA mRNA production and protein secretion but increases the production and secretion of plasminogen activator inhibitor 1, the major natural inhibitor of t-PA *in vivo*. If, as is suggested by this work, raised intraluminal pressure, by increasing circumferential wall strain, impairs the capacity for acute endothelial t-PA release *in vivo*, this would provide a direct mechanistic link between raised blood pressure and atherothrombotic events. It might further be hypothesized that the clinical benefit of blood pressure reduction in reducing these events would, at least in part, be mediated through improvements in endogenous fibrinolysis. Indeed, there is now good evidence that the clinical benefit of antihypertensive therapy is predominantly related to blood pressure reduction *per se* rather than being the result of other drug class-specific effects [5]. Therefore, improvement in endothelial t-PA release might be a common mechanism through which different antihypertensives reduce cardiovascular events.

Of the agonists known to stimulate endothelial t-PA release, bradykinin is one of the most potent. Bradykinin is largely metabolized by angiotensin-converting enzyme (ACE) and ACE inhibitors potentiate bradykinin-induced t-PA release. Indeed, infusion of enalaprilat stimulates t-PA release and this effect is mediated through endogenous bradykinin [6]. Thus, the established clinical benefit of ACE inhibitors in a range of cardiovascular conditions may be partly related to effects on endothelial t-PA release through preservation of endogenous bradykinin. In patients with hypertension, ACE inhibitors are no more effective than other antihypertensive classes in reducing cardiovascular events [7]. This may suggest that any benefit that arises through improved endothelial t-PA release with ACE inhibition is predominantly due to blood pressure reduction rather than specific ACE inhibitor-mediated effects on endogenous bradykinin. Despite this, it is intriguing to speculate that patients with drug-resistant hypertension might benefit specifically from an ACE inhibitor even in the absence of blood pressure reduction.

There are some limitations to the work performed both *ex vivo* and *in vitro* on the effect of mechanical forces on

endothelial t-PA production and release. The effect of strain on HAECs was only investigated at 10% stretch. This is thought to correspond to an intraluminal pressure of 170 mmHg in medium-sized arteries. However, endothelial cells in arteries are exposed to cyclical circumferential strain under normal circumstances and it is possible that, even at this level, t-PA production and release is inhibited. Thus, the difference between the effects of normal physiological strain and the greater strain associated with hypertension may be more relevant. Furthermore, neither model can be expected to reflect accurately the complex pressure-related stresses on the arterial wall that occur in hypertension. Indeed, these stresses will vary both with the vessel size and the subtype of hypertension, whether diastolic, systolic or mixed. In particular, the increased stiffness of large arteries that characterizes isolated systolic hypertension will limit the change in vessel diameter and therefore stretch on the endothelial cell layer. As a result, despite high systolic pressures, endothelial t-PA release might be relatively preserved.

It is now 7 years since Jern *et al.* [3] first described the important observation that endothelial t-PA release is impaired in patients with hypertension. However, many questions remain. For example, is the relationship between blood pressure and t-PA release linear? What are the effects of the continuous and pulsatile components of blood pressure? Is there a regional or systemic impairment of t-PA release? Does blood pressure reduction improve t-PA release? Are there differences between antihypertensive classes with respect to improving t-PA release? Answering these and other, related, questions may significantly contribute to our understanding of how raised blood pressure leads to atherothrombotic events and why antihypertensive therapy effectively reduces their incidence.

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**PUBLICATION 35**

## Review

## Clots, kinins and coronaries

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**Abstract**

The dynamic regulation of intravascular thrombus formation is central to our understanding of both acute and chronic atherosclerotic events. The initiation, modification and resolution of thrombus associated with eroded or unstable coronary plaques is critically dependent on the efficacy of endogenous fibrinolysis, a process that is itself reliant upon the cellular activation and function of the surrounding endothelium and vascular wall. Bradykinin is a vasodilator peptide that stimulates the endothelium to release the pro-lytic factor, tissue-type plasminogen activator and is released at sites of intravascular thrombus formation including the luminal surface of ruptured or eroded atheromatous plaques. Recent studies have provided important and novel insights into the contribution of bradykinin to the regulation of endogenous fibrinolysis and intravascular thrombosis in the peripheral and coronary circulations in vivo in man. Moreover, the pro-fibrinolytic effects of bradykinin are markedly augmented in the presence of angiotensin-converting enzyme inhibition and may explain, at least in part, the established anti-ischaemic effects of angiotensin-converting enzyme inhibitors in patients with atherosclerosis. The development of novel agents that potentiate bradykinin and endogenous fibrinolysis, such as inhibitors of thrombin activatable fibrinolysis inhibitor, may provide future therapeutic strategies to treat and prevent cardiovascular disease.

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**Keywords:** Atherosclerosis; Angiotensin-converting enzyme; Bradykinin; Endogenous fibrinolysis; Endothelium; Tissue-type plasminogen activator**Contents**

1. Bradykinin .....	190
2. Bradykinin is an endothelium-dependent vasodilator .....	191
3. Endogenous fibrinolysis .....	192
4. Bradykinin stimulates endothelial release of t-PA .....	192
5. Mechanism of bradykinin-mediated vasodilatation and t-PA release .....	192
6. Bradykinin and angiotensin-converting enzyme inhibition .....	192
7. Bradykinin, angiotensin II and plasminogen activator inhibitor type-1 .....	193
8. Bradykinin and angiotensin receptor blockade .....	193
9. Bradykinin, angiotensin-converting enzyme inhibition and inflammation .....	193
10. Clinical relevance .....	194
11. Novel therapeutic strategies .....	194
11.1. Inhibition of neutral endopeptidase .....	194
11.2. Inhibition of thrombin activatable fibrinolysis inhibitor .....	194
12. Summary .....	195
References .....	195

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each with high affinity and specificity for their respective ligands, des-Arg<sup>9</sup>-bradykinin and bradykinin [17]. The B<sub>1</sub> and B<sub>2</sub> kinin receptors share only 36% homology with each other and are distinguishable on the basis of their pharmacological and molecular characteristics [17–19]. The B<sub>2</sub> receptor is the most prevalent receptor subtype expressed on the vascular endothelium and smooth muscle under physiological conditions. In contrast, the vascular B<sub>1</sub> receptor is normally expressed very weakly but is rapidly upregulated, at least in animal models and in vitro in human tissue, in the presence of inflammation [20–23], ischaemic left ventricular dysfunction [24], cardiovascular disease [22,25,26] and ACE inhibition [27,28]. In addition, intense endothelial B<sub>1</sub> receptor expression has been demonstrated in human atheromatous plaques at autopsy [26].

## 2. Bradykinin is an endothelium-dependent vasodilator

In animal studies, the haemodynamic effects of bradykinin vary depending on the species and tissue being studied and the dose of bradykinin applied. In mammals, intravenous administration of bradykinin results in arteriolar vasodilatation and a rapidly reversible fall in blood pressure [12]. The short-lived nature of this hypotensive effect has been attributed to reflex chronotropic and inotropic responses [12]. In human coronary arteries in vitro, bradykinin causes endothelium-dependent vasorelaxation [29] and intravenous and intra-arterial injection of bradykinin at systemic doses in man results in a fall in blood pressure with a marked reduction in peripheral vascular resistance [16,30]. The arterio-venous difference in the dose of bradykinin producing a similar reduction in blood pressure suggests a pulmonary clearance rate for bradykinin of 95% [16].

At a local level, intra-arterial infusion of bradykinin causes endothelium dependent vasodilatation in the forearm (Fig. 3) [6,7,31] and coronary circulations [32,33]. Moreover, although the epicardial coronary response to bradykinin does not appear to correlate with presence of risk factors for atherosclerosis [30], bradykinin-mediated vasodilatation within the human coronary circulation is impaired at sites where atherosclerotic plaque is present [34].

Bradykinin does not appear to contribute to basal vascular tone in the human forearm or systemic circulations under normal physiological conditions. In the human forearm circulation, intra-brachial administration of the bradykinin B<sub>2</sub> receptor antagonist, HOE-140, also known as icatibant, has no effect on resting blood flow [35–37]. Likewise, systemic administration of HOE-140 has no effect on blood pressure in healthy volunteers [38]. In contrast, however, there is evidence that bradykinin contributes to the regulation of basal vascular tone in the coronary circulation in man [39]. Intra-coronary administration of HOE-140 increases coronary vascular resistance and reduces coronary blood flow

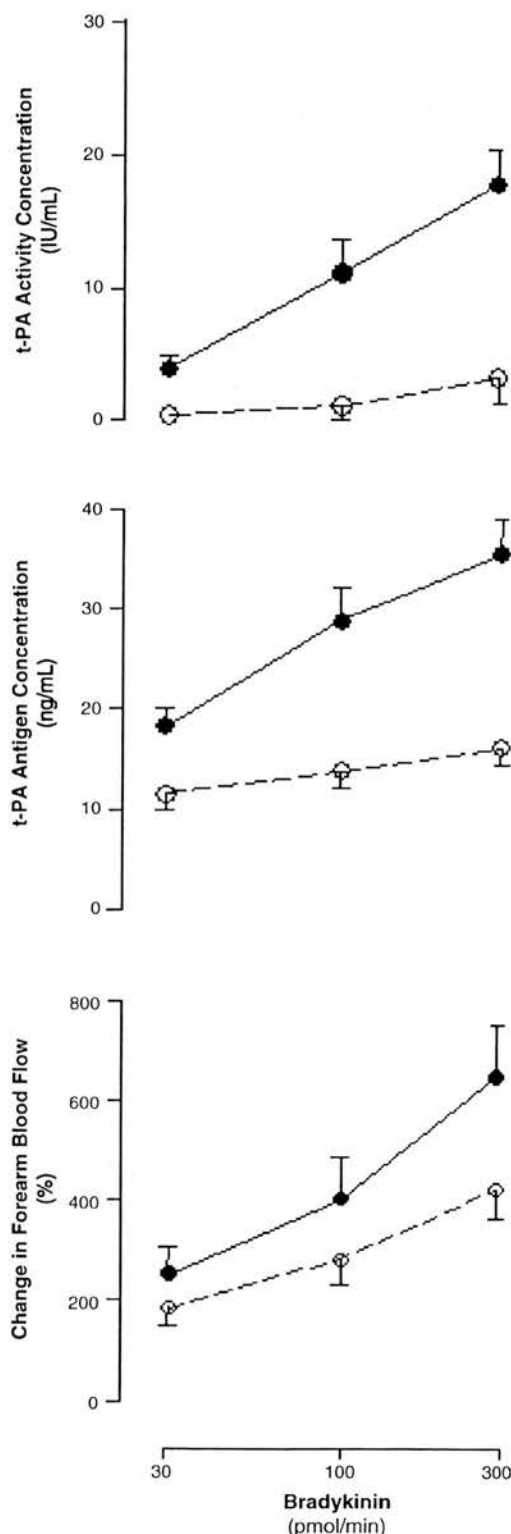


Fig. 3. Effect of intra-arterial bradykinin on blood flow and venous tissue-type plasminogen activator and activity concentration in the forearm circulation of patients with heart failure treated with enalapril 10 mg twice daily for 5 weeks (closed circles) and following ACE inhibitor withdrawal (open circles;  $p < 0.001$ , open vs. closed circles for all, ANOVA) [50].

under basal conditions and during flow-associated vasodilatation [39].

### 3. Endogenous fibrinolysis

The endogenous fibrinolytic system describes a complex process ultimately resulting in the hydrolytic cleavage of fibrin by plasmin to cause clot dissolution and generate fibrin degradation products. The major role of this enzymatic system is to protect the circulation from intravascular fibrin formation and thrombosis that would otherwise result in vessel occlusion and tissue ischaemia. The main physiological plasminogen activator involved in the degradation of intravascular fibrin in man is tissue-type plasminogen activator (t-PA) [40,41].

Tissue-type plasminogen activator is released from the endothelium through the translocation of a dynamic intracellular storage pool [42] and the efficacy of plasminogen activation and fibrin degradation is determined by the relative balance between the acute local release of t-PA and its subsequent inhibition through formation of complexes with the serpin, plasminogen activator inhibitor type 1 (PAI-1).

### 4. Bradykinin stimulates endothelial release of t-PA

Bradykinin is a potent stimulus for t-PA release from endothelial cells in vitro and in animal models in vivo [42–44]. Subsequent work by Brown et al. demonstrated an increase in venous t-PA antigen concentrations following intravenous administration of bradykinin in human subjects treated with ACE inhibition [30]. Systemic bradykinin infusion, however, was associated with a significant fall in blood pressure and an associated increase in heart rate [30]. As a result, the authors could not exclude the possibility that plasma t-PA concentrations rose as a result of concomitant neurohumoral and haemodynamic effects, in particular adrenergic stimulation [30,45].

Combining venous occlusion plethysmography with bilateral venous sampling led to the development of a novel in vivo model for assessment of endogenous t-PA release in the human forearm circulation in response to specific pharmacological stimuli in the absence of confounding systemic responses [46]. Using this powerful pharmacological model, we and others have demonstrated that bradykinin stimulates endothelial t-PA release in the human forearm circulation in a dose-dependent fashion (Fig. 3) without affecting plasma concentrations of PAI-1 or von Willebrand factor [6,47,48]. Given the absence of an increase in t-PA with the vasodilator, sodium nitroprusside, this effect could not be attributed to local changes in blood flow [6,47]. Similarly, in the human coronary circulation, bradykinin stimulates endothelial release of t-PA without affecting plasma concentrations of PAI-1 [49].

### 5. Mechanism of bradykinin-mediated vasodilatation and t-PA release

In the human forearm circulation, bradykinin-induced vasodilatation and endothelial t-PA release are both mediated via the B<sub>2</sub>, but not the B<sub>1</sub>, kinin receptor [9,50,51]. Downstream signalling mechanisms differ between vasodilatation and t-PA release, as well as the vascular bed being studied.

In the human forearm circulation, high K<sup>+</sup> concentrations [52] and inhibitors of K<sup>+</sup> transport across the vascular smooth muscle cell membrane [52–55], but not inhibitors of nitric oxide synthase or prostaglandin synthesis [55], attenuate bradykinin-mediated endothelium dependent vasodilatation suggesting a role for an endothelium-derived hyperpolarising factor. In contrast, in the coronary circulation in vivo in man, inhibition of nitric oxide synthase attenuates the bradykinin-mediated increase in coronary artery diameter and blood flow [32].

The mechanism by which bradykinin induces t-PA release downstream of the B<sub>2</sub> kinin receptor is less clear. As with bradykinin-mediated vasodilatation, inhibition of nitric oxide synthase and prostaglandin synthesis does not attenuate the bradykinin-induced increase in t-PA release in the human forearm circulation in vivo [9]. The effects of inhibitors of endothelium-derived hyperpolarising factor on bradykinin-mediated t-PA release are not known.

### 6. Bradykinin and angiotensin-converting enzyme inhibition

Angiotensin-converting enzyme (ACE) inhibitors have become a cornerstone in the management of a number of cardiovascular diseases including atherosclerosis and congestive cardiac failure due to left ventricular systolic dysfunction. In man inhibitors of ACE cause peripheral vasodilatation [56], reduce peripheral vascular tone and systemic arterial pressure [56], reverse endothelium-dependent vasomotor dysfunction [57,58] and increase plasma t-PA concentrations [59,60]. There is now convincing evidence that the haemodynamic and fibrinolytic effects of ACE inhibitors are, at least in part, due to bradykinin [38,60,61].

Although the short half-life of bradykinin in plasma makes accurate measurement difficult, elevated plasma bradykinin concentrations have been reported in the presence of ACE inhibition [62]. In man, ACE inhibition enhances flow-mediated dilatation of the brachial artery by a bradykinin B<sub>2</sub> receptor-dependent mechanism [37] and augments bradykinin-mediated vasodilatation and endothelial t-PA release in the forearm [7,35,47,63,64] (Fig. 3) and coronary [32,49,65] circulations.

An insertion deletion (I/D) polymorphism in intron 16 of the ACE gene accounts for ~50% of the variability in human serum ACE levels [66]. Angiotensin-converting enzyme genotype has been shown to modulate the rate of

bradykinin metabolism both *in vitro* [67] and *in vivo* [68]: the D allele being associated with increased ACE activity [69]. Whilst the relationship between ACE genotype and the vasodilator response to bradykinin appears complex and may be related to ethnicity [70,71], the D/D ACE genotype has been associated with impaired bradykinin-induced t-PA release in the human coronary circulation [72].

In healthy volunteers, ACE inhibition with quinapril increases bradykinin induced t-PA release 2-fold in comparison to treatment with placebo or the angiotensin receptor blocker, losartan [47]. This is in marked contrast to the 10-fold increase in bradykinin-induced t-PA release observed in patients with heart failure due to ischaemic heart disease during ACE inhibitor treatment compared to its withdrawal (Fig. 3) [64]. This massive augmentation of t-PA release leads to local plasma t-PA concentrations that approach those seen during systemic fibrinolytic therapy for acute myocardial infarction [64]. These data underscore the vast reserves of intracellular t-PA present within the endothelium and highlight the significant pro-fibrinolytic potential of pharmacological strategies targeting this pathway.

Consistent with data from animal models [73], bradykinin receptor antagonism attenuates the haemodynamic effects of ACE inhibition in man. In salt deplete healthy volunteers as well as hypertensive subjects, Gainer et al. demonstrated that a systemic infusion of HOE-140 attenuates the hypotensive response to a single dose of captopril [38]. The reduction in blood pressure observed following co-administration of HOE-140 and captopril was similar to that seen with the angiotensin receptor blocker, losartan [38]. In patients with heart failure, the kinin receptor antagonist, B9340, caused dose-dependent vasoconstriction in the forearm circulation in the presence, but not absence of ACE inhibition [36]. Moreover, B9340 had a significant pressor effect when administered systemically in patients with heart failure treated with enalapril compared to losartan [61].

In addition to the haemodynamic effects, there is evidence that bradykinin contributes to the increase in constitutive t-PA release associated with ACE inhibitor therapy by a B<sub>2</sub> receptor-dependent mechanism. In the forearm circulation of healthy smokers, intra-arterial enalaprilat increased net basal t-PA release and this effect was blocked by pre-treatment with HOE-140 [60]. In this study the authors also note that ACE inhibition appears to augment bradykinin-mediated endothelial t-PA release to a greater extent than vasodilatation [60]. The reason for this discrepancy is not clear but a number of explanations have been proposed including alterations in bradykinin receptor sensitivity through direct ACE-receptor interactions [74] and modulation of post-receptor signalling. Moreover, these data emphasise the differences between downstream signalling mechanisms that regulate vascular tone and t-PA release discussed earlier.

## **7. Bradykinin, angiotensin II and plasminogen activator inhibitor type-1**

Besides alterations in plasma t-PA concentrations, ACE inhibitors favourably alter endogenous fibrinolytic balance through a reduction in plasma PAI-1 and the overall PAI-1:t-PA ratio [75–77]. Inhibitors of ACE block the production of angiotensin II in addition to increasing plasma bradykinin concentrations. Although bradykinin has no effect on PAI-1 concentrations, angiotensin II causes the release of PAI-1 *in vitro* [78] and in animal models *in vivo* [79]. In man, intravenous [80], but not intra-arterial [6], administration of angiotensin II increases plasma PAI-1 concentrations without affecting t-PA concentrations. It is likely, therefore, that in addition to potentiation of bradykinin, suppression of angiotensin II mediated PAI-1 release contributes to the pro-fibrinolytic effects of ACE inhibitor therapy.

## **8. Bradykinin and angiotensin receptor blockade**

There are data indicating that bradykinin may contribute to the vascular effects of angiotensin receptor blockers by an angiotensin II type 2 receptor-mediated mechanism. In transgenic mice overexpressing the AT<sub>2</sub> receptor, angiotensin II causes vasodilatation that is attenuated by HOE-140 [81]. Elevated plasma bradykinin concentrations have recently been reported in subjects with hypertension treated with losartan [82] and infusion of the bradykinin antagonist, HOE-140, inhibits the improvement in flow-mediated vasodilatation associated with candesartan in healthy volunteers [83]. In addition, in patients with heart failure treated with losartan, we observed a small increase in blood pressure following infusion of the bradykinin receptor antagonist, B9340, compared to placebo [61] lending further support to the hypothesis that bradykinin may contribute to the vascular effects of angiotensin receptor blockade.

## **9. Bradykinin, angiotensin-converting enzyme inhibition and inflammation**

Inflammation is thought to play a key role in the pathogenesis of atherosclerosis. There is increasing evidence that alterations in the inflammatory response may contribute to the clinical benefits observed with ACE inhibitor therapy. It remains unclear to what extent bradykinin, or indeed angiotensin II, contributes to this action. Both angiotensin II [84] and bradykinin [85] stimulate the release of interleukin-6. In contrast, in patients with heart failure high-dose enalapril therapy is associated with a significant decrease in interleukin-6 activity [86], whilst in patients undergoing coronary artery bypass grafting requiring cardiopulmonary bypass, ACE inhibitors but not angiotensin receptor blockers attenuate the associated interleukin-6 response [87]. These findings suggest that blockade of angiotensin II production



may be of greater importance than bradykinin accumulation in the anti-inflammatory properties of ACE inhibitor therapy. Further work is required to examine the contribution of bradykinin and angiotensin II to the anti-inflammatory effects of ACE inhibition and the relevance of this mechanism to atherothrombosis.

## 10. Clinical relevance

Detailed post-mortem studies have shown that plaque growth is induced by episodic subclinical plaque disruption and thrombus formation [88]. The prolonged presence of residual thrombus over a disrupted or eroded plaque will provoke smooth muscle migration and the production of new connective tissue, leading to plaque expansion [88]. Consistent with this, enhanced macrovascular fibrin deposition and atherogenesis are seen in genetic murine models of tissue-type plasminogen activator [89] and plasminogen [90] deficiency. However, in the presence of an imbalance in the fibrinolytic system, subclinical microthrombi on the surface of atherosclerotic plaques may propagate and ultimately lead to arterial occlusion and tissue infarction [91]. Indeed, reduced plasma fibrinolytic activity has been associated with an increased risk of myocardial infarction in both healthy individuals [92] and patients with cardiovascular disease [93]. Thus, the initiation, modification and resolution of thrombus associated with eroded and unstable plaques may be critically dependent on the efficacy of endogenous fibrinolysis.

There is now a clear link between impaired endothelial release of t-PA and risk factors for the progression of atherosclerosis. In man, the capacity of the coronary endothelium *in vivo* to release t-PA in response to acute stimulation with the neurokinin, substance P, is negatively associated with increasing coronary atheromatous plaque burden and smoking habit [94]. Moreover, both bradykinin and substance P-mediated endothelial t-PA release are impaired in the forearm circulation of cigarette smokers [95,96]. Given that the vasomotor effects of bradykinin in the human coronary circulation are attenuated at sites of atheromatous plaque [34], it is likely that bradykinin-mediated endothelial t-PA release may also be impaired.

Bradykinin is released during the contact phase of coagulation (Fig. 1) [10]. In clinical studies of patients with unstable angina, activation of the kallikrein system is enhanced and bradykinin release is increased [97]. Given that bradykinin is a potent stimulator of endogenous t-PA release, this local liberation of bradykinin in acute coronary syndromes may represent an important negative feedback loop by which bradykinin-induced endothelial t-PA release inhibits thrombus formation within the vascular lumen. Moreover, this process is further amplified by the action of plasmin itself that, in combination with kallikrein, augments bradykinin release from high-molecular weight kininogen [98].

The major clinical benefits of ACE inhibition have been incontrovertibly established in patients with coronary artery disease. Although first demonstrated in patients with heart failure, it is now clear from the HOPE [99] and EUROPA [100] trials that ACE inhibitor therapy benefits all patients with cardiovascular disease. Indeed, when comparing these studies with a meta-analysis of heart failure trials [101] there is a clear and remarkably consistent anti-ischaemic effect of ACE inhibitors with a 20–22% relative risk reduction in the rate of myocardial infarction. In support of this hypothesis, ACE inhibitor therapy reduces myocardial troponin release in patients with acute coronary syndromes [102]. In contrast, the beneficial effects on mortality appear to depend upon the overall cardiovascular risk of the patient: greatest in those with severe left ventricular dysfunction and least in those at low risk with preserved left ventricular dysfunction. Thus, both the augmentation of bradykinin-induced vasodilatation and the marked increase in t-PA release described with ACE inhibition in patients with ischaemic heart disease may contribute to the primary mechanism of the anti-ischaemic effects associated with chronic ACE inhibitor therapy.

## 11. Novel therapeutic strategies

### 11.1. Inhibition of neutral endopeptidase

Based on the success of ACE inhibitor therapy in the treatment of atherosclerotic vascular disease and heart failure, attention has focused on inhibiting alternative pathways of bradykinin breakdown in an effort to augment both the vasomotor and fibrinolytic actions of bradykinin.

Neutral endopeptidase (NEP) is a glycosylated metalloprotease that colocalises with ACE on the cell membrane [103]. Found in a wide variety of tissues including the vascular endothelium, NEP has a broad substrate specificity metabolising a number of biologically active peptides including endothelin, atrial natriuretic peptide, substance P and bradykinin [103]. In the presence of ACE inhibition, the contribution of NEP to bradykinin metabolism is increased [104]. We have recently demonstrated in patients with heart failure that combined ACE and NEP inhibition augments the vasomotor and fibrinolytic effects of bradykinin compared to ACE inhibition alone [51]. Although a recent large-scale trial failed to demonstrate a significant treatment benefit with combined ACE and NEP inhibition in heart failure [105], the role of combined ACE and NEP inhibition in the management of atherosclerotic vascular disease remains unclear.

### 11.2. Inhibition of thrombin activatable fibrinolysis inhibitor

The rate of removal of the C-terminal arginine from bradykinin is more rapid than can be attributed to carboxypeptidase N alone [106] and it is likely that other carboxypeptidases, in particular carboxypeptidase U, are



involved in this metabolic pathway [10]. Carboxypeptidase U hydrolyses bradykinin *in vitro* as efficiently as it does plasmin cleaved fibrin peptides [107]. Although the contribution to bradykinin metabolism *in vivo* remains to be determined, carboxypeptidase U has additional properties that allow it to modulate the fibrinolytic effects of bradykinin and these will be discussed below.

Carboxypeptidase U is a recently described enzyme that is present in human plasma, circulates as a zymogen and is activated by thrombin and plasmin [108]. In addition to its effects on bradykinin metabolism, it also has a role in the regulation of endogenous fibrinolysis and gives rise to its other more commonly used name, TAFI [108]. Thrombin activatable fibrinolysis inhibitor acts through cleavage of the lysine residues on fibrin and thereby prevents the binding of plasminogen to the fibrin clot [108]. This greatly reduces the ability of t-PA to cleave plasminogen to plasmin and attenuates fibrinolysis. Thus, TAFI inhibits clot lysis in the presence of high thrombin concentrations.

Several TAFI inhibitors are currently under development and may lead to potentially novel and important therapies in the treatment and prevention of coronary artery disease [109,110]. Potato carboxypeptidase inhibitor (PCI) is a 39 amino acid peptide derived from potato tubers [109] and is a specific inhibitor of activated TAFI [111,112]. In a canine model of acute myocardial infarction, TAFI inhibition with PCI produced a more rapid reperfusion of the infarct-related artery during thrombolytic therapy [112]. Using thromboelastography, we have recently demonstrated that PCI potentiates t-PA induced fibrinolysis *in vitro* in whole human blood [113]. Inhibitors of TAFI, therefore, may not only render intravascular thrombus more susceptible to the actions of t-PA but also up regulate bradykinin-induced pathways of endothelial t-PA release. To date, however, the effects of TAFI inhibition both on bradykinin metabolism and t-PA mediated fibrinolysis *in vivo* in man are unknown.

On a more cautious note, it should be noted that potentiating the vascular actions of bradykinin may have detrimental effects. Bradykinin has been implicated in the pathogenesis of ACE inhibitor-mediated angioedema [114]. Compared to ACE inhibition an even greater incidence of angioedema has been reported following treatment with combined ACE and NEP inhibition [115]. Further work is required to establish the role of bradykinin in the pathophysiology of this potentially life threatening condition.

## 12. Summary

The dynamic regulation of intravascular thrombus formation is central to our understanding of both acute and chronic atherosclerotic events. It has become clear that endogenous fibrinolysis and t-PA play a key role in the initiation, modification and resolution of thrombus associated with eroded or unstable coronary plaques. Bradykinin is a vasodilator peptide produced at sites of intravascular thrombus formation

that stimulates the endothelium to release t-PA. Recent studies have highlighted the potential importance of bradykinin in the regulation of endogenous fibrinolysis and intravascular thrombosis *in vivo* in man. Inhibitors of ACE markedly augment both the vasomotor and pro-fibrinolytic effects of bradykinin and this may explain, at least in part, the potent anti-ischaemic effects of ACE inhibitors in patients with atherosclerosis. The development of novel agents that potentiate bradykinin and endogenous fibrinolysis, such as inhibitors of TAFI, may provide future therapeutic strategies to treat and prevent cardiovascular disease.

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**PUBLICATION 36**



## ATVB In Focus

### Noninvasive Assessment of Atherosclerosis: From Structure to Function

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## Stimulated Tissue Plasminogen Activator Release as a Marker of Endothelial Function in Humans

James J. Oliver, David J. Webb, David E. Newby

**Abstract**—The initiation, modulation, and resolution of thrombus associated with eroded or unstable coronary plaques are critically dependent on the efficacy of endogenous fibrinolysis. This is dependent on the cellular function of the surrounding endothelium and vascular wall. In particular, the acute release of tissue plasminogen activator from the endothelium makes an important contribution to the defense against intravascular thrombosis. Here, we describe the rationale and methodology for, and clinical relevance of, assessing acute endothelial tissue plasminogen activator release in humans. The investigation of endothelial fibrinolytic function has the potential to provide major new insights into the pathophysiology of cardiovascular disease, and to shape future therapeutic interventions. (*Arterioscler Thromb Vasc Biol.* 2005;25:2470–2479.)

**Key Words:** arterial thrombosis ■ endothelial function ■ endothelium ■ fibrinolysis ■ thrombosis

The endothelium plays a vital role in the control of blood flow, coagulation, fibrinolysis, and inflammation. To date, clinical studies have focused on the assessment of endothelium-dependent vasomotion as a surrogate measure of endothelial function, and there is now extensive evidence of abnormal endothelium-dependent vasodilatation in patients with atherosclerosis and its associated risk factors.<sup>1</sup> Abnormal vasomotor responses independently predict cardiovascular events.<sup>2</sup> However, endothelium-dependent vasomotion may not be representative of other important aspects of endothelial function, such as the regulation of fibrinolysis.

In health, the endothelium prevents thrombus formation through a number of mechanisms. Thrombomodulin, protein S, heparan sulfate proteoglycans, and tissue factor pathway inhibitor are all endothelium-derived inhibitors of coagulation, whereas prostacyclin, nitric oxide (NO), and surface-bound CD39 inhibit platelet aggregation. However, when endothelial function is perturbed, for example with injury or inflammation, it can rapidly become procoagulant by downregulating its anticoagulant functions, inducing tissue factor expression and increasing secretion of factors such as fibronectin, von Willebrand factor (vWF), and platelet activating factor.<sup>3</sup>

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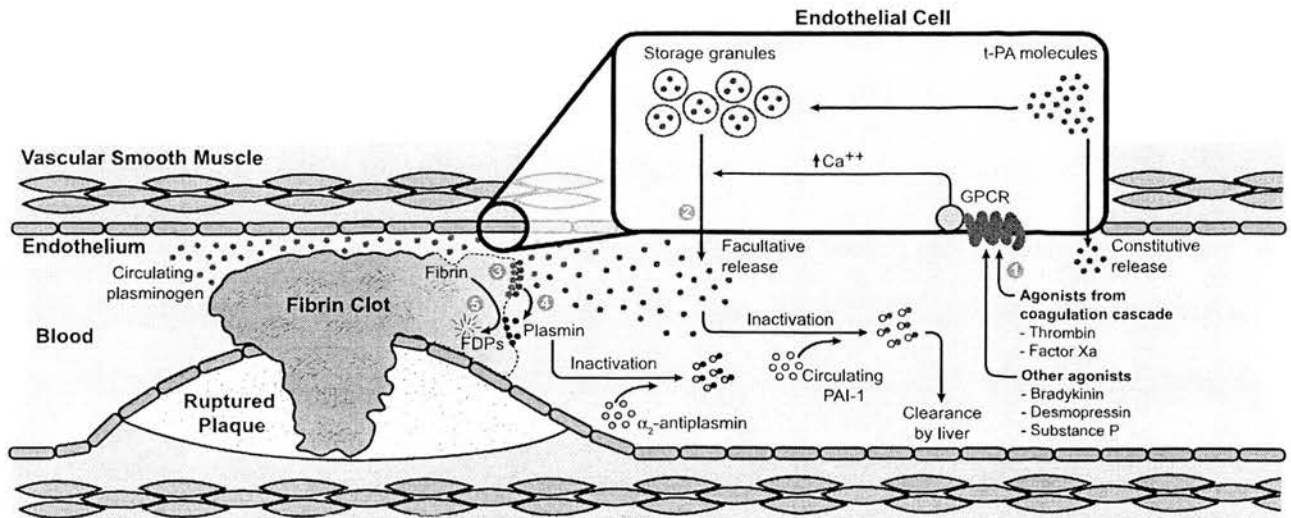
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**Figure 1.** The endothelial fibrinolytic response to luminal thrombus. Agonists generated from the coagulation cascade act on endothelial cell surface G protein-coupled receptors (GPCRs) (1) to stimulate release of tissue plasminogen activator (t-PA) from storage granules, a step that requires an increase in intracellular calcium ( $\text{Ca}^{++}$ ) concentration (2). Free t-PA acts on thrombus-bound plasminogen (3) to produce plasmin (4) that, in turn, degrades cross-linked fibrin into fibrin degradation products (FDPs) (5), thus dissolving the thrombus. The fibrinolytic process is inhibited by inactivation of t-PA by PAI-1 and plasmin by  $\alpha_2$ -antiplasmin.

### Endogenous Fibrinolysis

The endogenous fibrinolytic system protects the circulation from intravascular fibrin formation and thrombosis. After initiation of thrombus formation, the endothelium acutely releases tissue plasminogen activator (t-PA) in response to a range of factors predominantly related to the coagulation cascade, especially factor Xa and thrombin.<sup>4</sup> Once released, t-PA catalyzes the conversion of plasminogen to plasmin, facilitating thrombus dissolution through the proteolytic degradation of fibrin to soluble fibrin degradation products (Figure 1). The conversion of plasminogen to plasmin by t-PA is accelerated in the presence of fibrin and at the endothelial cell surface,<sup>5,6</sup> ensuring efficient localized activation. Because plasminogen is present at vast molar excess over t-PA in plasma, the onset and efficacy of fibrinolysis are principally determined by the rapidity and magnitude of t-PA release.

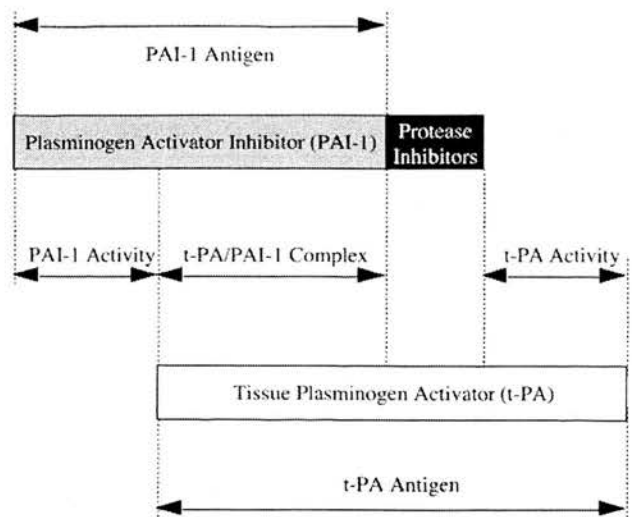
The concentration of t-PA in human plasma is  $\approx 3$  to 10 ng/mL, but a relatively small proportion is functionally active because of the presence of serine protease inhibitors (serpins); principally, plasminogen activator inhibitor (PAI) type 1 (PAI-1), but also PAI-2, PAI-3,  $\alpha_2$ -macroglobulin, and C1 esterase inhibitor (Figure 2).<sup>7</sup> The proportion of active t-PA varies inversely with plasma PAI-1 concentration, from 2% to 33%.<sup>8</sup> The plasma half-life of t-PA is  $\approx 5$  minutes, and the liver is the major site of clearance.<sup>9</sup> The interaction between t-PA and PAI-1 has a rapid second order rate constant of  $\approx 10^7 \text{ M}^{-1} \text{ s}^{-1}$ ,<sup>10</sup> and there is a several-fold molar excess of PAI-1 over t-PA in plasma.<sup>8</sup> Therefore, for active unbound t-PA to reach a thrombus, rapid local release is vital, particularly because fibrinolysis is much more effective if t-PA is incorporated during, rather than after, thrombus formation.<sup>11</sup>

### Synthesis, Storage, and Release of t-PA

Encoded by a gene on chromosome 8, t-PA is a 68 kDa serine protease of 530 amino acids and the endothelium is its

principal site of generation. Endothelial cells in culture synthesize and constitutively secrete t-PA.<sup>12</sup> The rate of synthesis is increased by a number of substances, including thrombin and histamine,<sup>13</sup> and is reduced by plasmin.<sup>14</sup> However, although protein kinase C appears to play a role,<sup>15</sup> the mechanisms regulating t-PA synthesis have not been characterized in detail.

t-PA is released facultatively from storage granules and the pathways of constitutive and facultative release differ.<sup>12</sup> Some workers have suggested that t-PA is stored with vWF in Weibel-Palade bodies,<sup>16</sup> but there is now convincing evidence that t-PA is stored in vesicles distinct from Weibel-Palade bodies,<sup>17,18</sup> and this is consistent with the *in vivo* observation



**Figure 2.** Relationship between tissue plasminogen activator (t-PA) and its major inhibitor, plasminogen activator inhibitor type 1 (PAI-1), in plasma. Only the unbound fractions are active. Other protease inhibitors, including PAI-2, PAI-3,  $\alpha_2$ -macroglobulin, and C1 esterase inhibitor, also bind to t-PA in plasma.

**Substances That Stimulate Acute Tissue Plasminogen Activator (t-PA) Release in Humans**

	t-PA Release	Mechanism	Dose	Comments	References
Bradykinin	++++	B <sub>2</sub> receptor-dependent NO and prostaglandin- independent	0.02 to 3 nmol/min	Bradykinin-induced t-PA release potentially relevant to contact phase of the intrinsic coagulation pathway and the action of ACE inhibitors	20, 28, 40, 42, 51, 52, 54, 55, 57, 91, 93, 98, 99
Substance P	+++	NK <sub>1</sub> receptors	2 to 40 pmol/min		43, 53
TNF- $\alpha$	+++	Uncertain	80 to 240 ng/min	t-PA release has delayed onset and is sustained for $\geq 3$ hours after infusion is stopped. May induce de novo t-PA protein synthesis.	73
Desmopressin	++	V <sub>2</sub> receptors	21 to 70 ng/min		26, 33, 48, 60, 95
Isoprenaline	++	Adrenergic receptors	400 ng/min		47
ATP	++	P2X or P2Y receptors	10 to 200		67
UTP		P2Y receptors	nmol/min		
Methacholine	+	Muscarinic receptors	0.8 to 12.8 $\mu$ g/min		26, 33, 46, 47, 62, 93
Norepinephrine	+	Adrenergic receptors	1.2 $\mu$ g/min		41

that agents stimulating t-PA release do not also release vWF.<sup>19,20</sup> The precise signaling pathways regulating facultative secretion have not been elucidated, but G-proteins and increased intracellular calcium concentrations appear to be important.<sup>18,21</sup>

Endothelial synthesis of t-PA varies with vessel size and anatomic location. In humans, immunoreactive t-PA is present in normal endothelium of the internal mammary and coronary arteries, saphenous vein, and aorta.<sup>22–24</sup> Release of t-PA also varies with region; the upper limbs, for example, releasing more than the lower limbs.<sup>25</sup> The capacity of the endothelium to store and release t-PA is substantial: continuous t-PA release can be induced for many hours without undergoing significant tachyphylaxis<sup>26,27</sup> and the forearm can release up to 4.5  $\mu$ g/min,<sup>28</sup> enough for local plasma concentrations to approach those achieved during systemic therapeutic thrombolysis.

**Acute Endothelial t-PA Release****In Vitro and Ex Vivo Assessment**

The development of relatively simple models of acute t-PA release has enabled much of the research on facultative t-PA release to be performed in vivo in humans. However, investigation of the relevant cellular and molecular pathways requires alternative approaches including cell culture and ex vivo animal models. Endothelial cell culture techniques have limitations and may not truly represent the in vivo function of these cells. Release of t-PA in cultured cells is slow and modest, and prolonged incubation periods and sensitive t-PA assays are required. Consequently, assessment of acute t-PA release is difficult to achieve in vitro. Moreover, the phenotype of cultured endothelial cells, including their ability to release t-PA, also changes with increasing passages. Ex vivo animal models, which provide a more favorable volume to surface area ratio, have been used to investigate various stimulants of acute t-PA release, for example, hydrogen peroxide in isolated rat heart.<sup>29</sup> The demonstration that agonist-stimulated t-PA release in rat hind limb did not require de novo protein synthesis<sup>30</sup> led to the concept that

acute release results from translocation of a dynamic intracellular storage pool<sup>12</sup> in response to blood coagulation and humoral factors.<sup>4</sup>

**In Vivo Assessment**

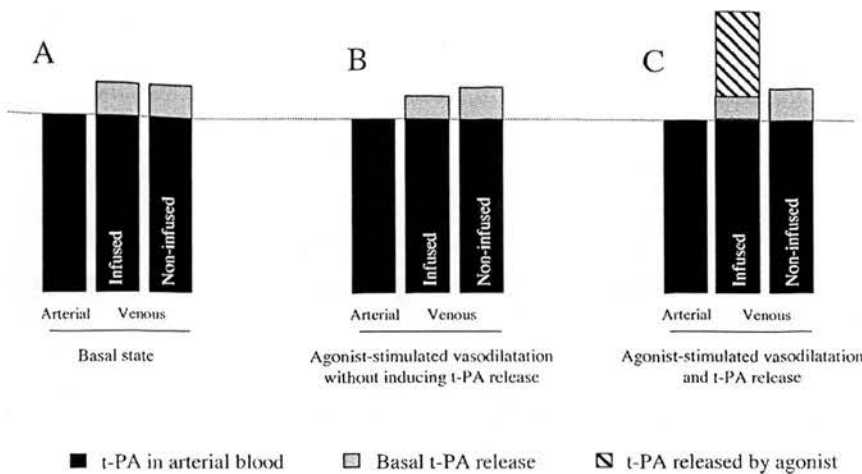
In humans, acute release of t-PA can be assessed systemically, for example after intravenous infusion of desmopressin<sup>31</sup> or bradykinin.<sup>32</sup> However, this approach is limited by potential confounding effects, such as changes in systemic hemodynamics, clearance of t-PA and PAI-1, activation of the sympathetic nervous system, and concomitant release of other mediators. Direct assessment of local capacity for acute t-PA release within individual vascular beds avoids these problems and is likely to better represent the defense against arterial thrombosis. Local availability of active t-PA depends on the extent of local t-PA release rather than on the amount of t-PA or PAI-1 entering the tissue in arterial blood.<sup>33</sup>

**Venous Occlusion Test**

Regional t-PA release can be assessed in vivo by measuring the increase in t-PA concentration in pooled venous plasma. Typically, an upper arm or leg cuff is inflated to between systolic and diastolic blood pressure to cause venous pooling. Blood is sampled before and 10 or 20 minutes after cuff inflation.<sup>34</sup> Using this methodology, reduced t-PA release has been reported in various groups of subjects, including those who smoke,<sup>35</sup> although there are conflicting data on patients with coronary artery disease.<sup>36,37</sup> Whereas the increase in t-PA in pooled venous blood may simply be because of continued secretion at the basal rate,<sup>25</sup> it has been suggested that venous occlusion itself stimulates t-PA release.<sup>38</sup> Although quite simple and widely applicable, the venous occlusion technique is a rather blunt tool with relatively poor reproducibility.<sup>39</sup>

**Regional Tissue Release**

Acute t-PA release has been assessed in both the forearm and coronary circulations of humans using a number of endothelial stimulants including bradykinin, substance P, desmopressin, and methacholine (Table).



**Figure 3.** Schematic comparison of measuring forearm t-PA release using the arteriovenous and venovenous techniques. A, Basal release of t-PA across the forearm can only be quantified with the arteriovenous technique. B, Through a dilutional effect, isolated vasodilation will reduce the concentration of t-PA in venous plasma in the infused arm. C, Agonists that induce t-PA release also commonly cause vasodilation, for example, bradykinin, substance P, and desmopressin. Under these circumstances the arteriovenous and venovenous techniques provide a similar measure of agonist-stimulated t-PA release. When the arteriovenous technique is used, basal release (determined before infusion of the agonist) should be subtracted from total release to derive agonist-induced release.

### Forearm Release

Two similar methodologies are used to assess forearm t-PA release in response to intrabrachial infusions: one based on the differences in plasma concentrations of t-PA between inflowing arterial and outflowing venous plasma of a single arm,<sup>40–42</sup> and the other based on the differences in venous plasma concentrations between the 2 arms.<sup>43</sup> For the arteriovenous technique, drugs are infused via an 18-gauge catheter and the arteriovenous concentration gradient is calculated from blood samples taken simultaneously from this and an ipsilateral venous catheter. Forearm plasma flow is calculated using forearm blood flow (FBF), measured by strain gauge plethysmography, and arterial hematocrit corrected for 1% trapped plasma. Net release is calculated as the product of the arteriovenous concentration gradient and forearm plasma flow:

$$\text{Net release} = (C_v - C_a) \times \text{FBF} \times [(101 - \text{hematocrit})/100]$$

where  $C_v$  and  $C_a$  are the venous and arterial concentrations, respectively. The antigen concentrations of t-PA are measured using enzyme-linked immunosorbent assays,<sup>44</sup> and net release is expressed as ng per 100 mL of forearm tissue per minute. The activity of t-PA is determined photometrically<sup>45</sup> and expressed as IU per 100 mL of forearm tissue per min.

Typical resting arteriovenous differences in t-PA concentrations in the forearm are only  $\approx 10\%$  of total venous concentration and basal constitutive release is  $\approx 0.4$  to  $1.3$  ng/100 mL of tissue/min.<sup>19,26,40,42,46–48</sup> There is no demonstrable release of PAI-1 across the forearm; therefore, t-PA activity increases in parallel with t-PA antigen concentration.<sup>19,46,48</sup> There is no consensus on whether it is better to measure t-PA antigen or activity. Whereas it is only unbound t-PA that is functionally active (Figure 2), ultimately the efficacy of endogenous fibrinolysis is determined by the magnitude of local t-PA release and the resultant t-PA activity at the site of thrombus. Whether venous plasma t-PA antigen or activity concentrations best reflect this dynamic process remains unclear and will in part depend on local tissue perfusion rates and plasma serpin concentrations. Consequently, both t-PA antigen and activity are often presented.

Using the venovenous technique, drugs are infused into the brachial artery via a 27-gauge needle. This is narrower than the catheter used in arteriovenous studies because it is not used for blood sampling. Net release is calculated in a similar manner, except that the concentration gradient is that between the venous plasma of the infused and noninfused arms. The reproducibility of agonist-induced t-PA release using the venovenous technique has been shown to be good.<sup>49</sup>

The arteriovenous and the venovenous approaches both have benefits and limitations. Net release is calculated directly in the arteriovenous technique but indirectly in the venovenous technique, potentially resulting in different estimates of stimulated t-PA release under certain conditions (Figure 3). Basal release cannot be calculated using the venovenous technique. However, because basal release constitutes only a small proportion of the overall venous plasma concentration, the venovenous technique does provide an accurate assessment of stimulated t-PA release. The arteriovenous method is technically more challenging and the larger-bore arterial catheter makes it less suitable for repeated studies within individuals. Moreover, the arterial catheter presents a larger thrombogenic surface that may itself stimulate the fibrinolytic system. Both techniques assume that there is no clearance of t-PA across the forearm. This is a reasonable assumption because the liver is the major site of clearance, although there are endothelial receptors capable of clearing t-PA from the circulation.<sup>50</sup>

### Cardiac Release

In the coronary circulation, dynamic t-PA release is assessed by infusion of agents into the left main<sup>51,52</sup> or left anterior descending coronary artery,<sup>53</sup> and samples are obtained simultaneously from the coronary sinus and either the aorta or femoral artery.<sup>51–54</sup> Sampling blood from the coronary sinus is appropriate only during the evaluation of the left ventricle and, particularly, during left anterior descending artery infusions. Care must be taken to ensure that catheters do not impede flow in the coronary sinus, because this may divert blood into the anterior cardiac or Thebesian veins. Net t-PA release is calculated in a similar manner to the forearm technique, but with blood flow measured using coronary



Doppler combined with either quantitative coronary angiography or intravascular ultrasound (IVUS).

#### **Stimulation of t-PA Release**

Although a number of humoral and coagulant factors cause t-PA release, other factors, including angiotensin II,<sup>20</sup> acetylcholine,<sup>40,52,55</sup> and atrial natriuretic peptide,<sup>56</sup> do not release t-PA, despite causing marked vasomotor effects.

Bradykinin is an inflammatory vasodilator peptide and a powerful stimulant of endothelial t-PA release. It acts via the B<sub>2</sub> receptor in an NO-independent and prostaglandin (PG)-independent fashion.<sup>57</sup> An endothelium-derived hyperpolarizing factor probably contributes to bradykinin-induced vasodilation,<sup>58</sup> although whether endothelium-derived hyperpolarizing factor contributes to t-PA release is not known.

The tachykinin vasodilator, substance P, acts mainly through the neurokinin type 1 (NK<sub>1</sub>) receptor. It is a central and peripheral neurotransmitter and mediates neurogenic inflammation.<sup>59</sup> Substance P induces t-PA release in both the forearm and coronary circulations, and is the most potent known stimulant of t-PA in humans.

The vasopressin analogue, desmopressin, is a V<sub>2</sub> receptor agonist that releases t-PA in the forearm circulation.<sup>26,48,60</sup> When administered systemically, it also induces vWF release.<sup>61</sup>

The muscarinic receptor agonist methacholine induces t-PA release in the forearm, but to a lesser degree than bradykinin<sup>62</sup> or desmopressin.<sup>26,33</sup> In contrast, acetylcholine, also a muscarinic agonist, does not stimulate t-PA release.<sup>40,52,55</sup> The reason for this difference is not clear, although varying potencies and stabilities of the respective compounds may be responsible. These agents also appear to provoke vasodilation via differing mechanisms with acetylcholine, but not methacholine, being largely mediated through NO production.<sup>63</sup>

The adrenergic agonists, norepinephrine<sup>41</sup> and isoprenaline,<sup>47</sup> both induce t-PA release in the forearm, as does mental stress, which is associated with adrenergic activation.<sup>19,41</sup> These data suggest a role for the sympathetic nervous system in controlling vascular t-PA secretion. In support of this, chemical sympathectomy in rats reduces basal plasma t-PA concentrations and agonist-induced t-PA release in isolated vessels.<sup>64</sup> In a porcine model, stimulation of cardiac sympathetic nerves causes coronary t-PA release.<sup>65</sup> Although norepinephrine from sympathetic nerve endings may act on the endothelium to release t-PA, there is also intriguing evidence that sympathetic neurons can themselves synthesize and release t-PA.<sup>66</sup>

Both adenosine and uridine triphosphates (ATP and UTP) stimulate t-PA release in the forearm, apparently independently of NO and PG pathways.<sup>67</sup> This is of particular interest because these nucleotides are released by activated platelets, endothelial cells and cardiomyocytes during ischemia. The doses of ATP and UTP administered<sup>67</sup> resulted in similar plasma concentrations of the nucleotides to those commonly observed in the context of ischemia. Moreover, coronary ischemia in the pig<sup>68</sup> was associated with increased t-PA release.

#### **Regulation of t-PA Release**

The role of NO in the mechanism of endothelial t-PA release is unclear. Sodium nitroprusside, a spontaneous NO donor, does not stimulate either synthesis or release in cultured endothelial cells or stimulate release *in vivo*.<sup>40,46–48,53,60,69</sup> However, substance P-induced t-PA release was inhibited by the NO synthase inhibitor, N<sup>G</sup> monomethyl-L-arginine (L-NMMA).<sup>27</sup> In contrast, bradykinin-induced t-PA release was unaffected by L-NMMA in one study<sup>57</sup> and increased by L-NMMA in another.<sup>70</sup> Thus, NO alone does not induce t-PA release, although it may play a permissive or synergistic role in stimulated t-PA release.

Shear stress is a well-characterized stimulus for t-PA secretion from cultured endothelial cells.<sup>71</sup> In *ex vivo* human conduit vessels, shear stress stimulates t-PA expression and increases its intracellular storage pool without stimulating its release.<sup>72</sup> Consistent with this, marked increases in blood flow during infusions of vasodilators such as sodium nitroprusside and papaverine do not cause *in vivo* t-PA release.<sup>51,52</sup> A permissive role of increased shear stress is also unlikely given that intrabrachial tumor necrosis factor- $\alpha$  induces t-PA release without changing blood flow,<sup>73</sup> and the vasoconstrictor, norepinephrine, releases t-PA while reducing flow.<sup>41</sup> Thus, blood flow and shear stress appear to regulate endothelial t-PA synthesis and storage but do not affect its acute release.

#### **Genetic Influences on t-PA Release**

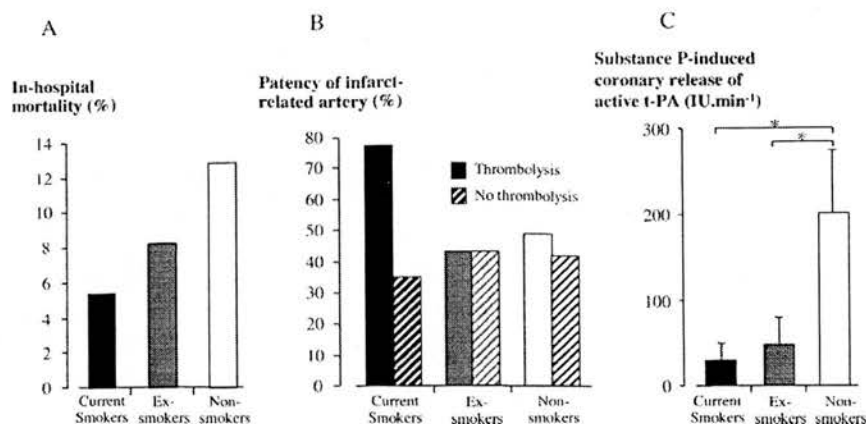
A number of small studies have investigated genetic influences on dynamic endothelial t-PA release. An Alu-repeat polymorphism and 3 single nucleotide polymorphisms in linkage disequilibrium with this<sup>74</sup> associate with t-PA release rates.<sup>74,75</sup> Although not apparent in the forearm circulation,<sup>55</sup> angiotensin-converting enzyme (ACE) I/D genotype may influence bradykinin-induced t-PA release in the coronary circulation.<sup>54</sup> In addition, there was no difference between healthy black and white Americans in bradykinin-induced t-PA release.<sup>55</sup> Further studies, with larger sample sizes, are desirable to confirm genetic linkage.

#### **Clinical Relevance of Endothelial t-PA Release**

Acute rupture or erosion of a coronary atheromatous plaque, and subsequent thrombosis, cause the majority of sudden cardiac deaths and myocardial infarctions.<sup>76</sup> Small areas of denudation and thrombus are commonly found on atheromatous plaques and are usually subclinical.<sup>77</sup> However, with imbalance in the fibrinolytic system, such microthrombi may propagate, leading to arterial occlusion.<sup>3</sup> The importance of acute endogenous t-PA release is further exemplified by the high rate of spontaneous reperfusion in the infarct-related artery after acute myocardial infarction.<sup>78</sup>

A reduction in t-PA activity or release is associated with an increased incidence of major adverse cardiac events in patients with stable<sup>79</sup> or unstable angina.<sup>36</sup> Rosenberg and Aird<sup>3</sup> have postulated that vascular bed-specific defects in hemostasis exist, and that coronary thrombosis critically depends on local fibrinolytic balance.





**Figure 4.** After myocardial infarction, smokers have a lower in-hospital mortality than nonsmokers (A) (data from Barbash et al<sup>100</sup>), possibly because thrombolytic therapy is more effective in smokers (B) (data from de Chillou et al<sup>87</sup>). Differences in the capacity for acute t-PA release in the coronary circulation (C) (data from Newby et al<sup>53</sup>) may explain this observation. Thrombus that develops despite normal endothelial cell t-PA release might be expected to be less susceptible to thrombolytic therapy than thrombus that develops in an artery with impaired capacity for endothelial t-PA release. \* $P \leq 0.03$ .

### Basal Plasma t-PA Concentrations

Several studies have investigated the relationship between basal venous t-PA antigen concentrations and subsequent coronary heart disease. In a meta-analysis of prospective studies, the risk of coronary heart disease was  $\approx 50\%$  greater in those with plasma t-PA antigen concentrations in the highest tertile compared with those in the lowest tertile.<sup>80</sup> This may seem counterintuitive but in part reflects the concomitant increase of plasma PAI-1 concentrations and associated reduction in t-PA activity. However, whereas this epidemiology is of interest, basal plasma t-PA does not reflect the local capacity for acute endothelial t-PA release in response to developing thrombus.<sup>33</sup> This underscores the importance of assessing acute endothelial t-PA release that is likely to be of greater pathophysiological relevance.

### Acute Coronary t-PA Release

The acute fibrinolytic activity of the heart is inversely correlated with the extent of proximal coronary artery atherosclerosis.<sup>53</sup> The mechanisms underlying this relationship are likely to involve chronic endothelial cell injury and impaired vascular function. Alternatively, this association may reflect chronic stimulation and upregulation of basal t-PA release secondary to atheroma and arterial denudation. The subsequent depletion of endothelial t-PA stores, and the desensitization and reduction of the acute fibrinolytic response, would potentially be detrimental.

Questions of cause and effect cannot be resolved by such observations and it remains possible that reduced fibrinolytic activity enhances atherogenesis. The prolonged presence of residual thrombus over a disrupted plaque will provoke smooth muscle migration and new connective tissue production, leading to plaque expansion.<sup>81</sup> This is consistent with the enhanced macrovascular fibrin deposition and atherogenesis seen in genetic murine models of t-PA deficiency.<sup>82</sup>

### Smoking

Basal plasma t-PA concentrations are either increased<sup>53,83</sup> or unaltered<sup>84</sup> in chronic smokers, but dynamic endothelial t-PA release is dramatically reduced. This has been consistently demonstrated in the forearm<sup>62,83</sup> and coronary circulations<sup>53</sup> (Figure 4), as well as with the venous occlusion test<sup>35</sup> and systemic desmopressin infusion.<sup>31</sup> The increased risk of spontaneous thrombosis in smokers<sup>85</sup> may, therefore, relate to

propagation of thrombus, which would otherwise undergo lysis and remain subclinical.

Although smokers have a higher overall mortality from myocardial infarction than nonsmokers,<sup>86</sup> their in-hospital mortality is lower (Figure 4).<sup>87</sup> This may, in part, be explained by the finding that in current smokers the infarct related artery is more likely to become patent after thrombolytic therapy.<sup>87</sup> These observations are consistent with these findings on endothelial t-PA release because it might be anticipated that patients with impaired endothelial cell t-PA release would benefit most from thrombolytic therapy, whereas those with a normal endogenous fibrinolytic capacity are more likely to have coronary thrombus resistant to fibrinolysis.

### Hypercholesterolemia

Patients with hypercholesterolemia have impaired endothelium-dependent vasodilatation. However, in contrast to smokers, hypercholesterolemia and lipid-lowering therapy do not influence acute t-PA release.<sup>49</sup> This is consistent with the finding that serum cholesterol concentrations, unlike smoking status, do not influence the patency rate of the infarct related artery after thrombolytic therapy.<sup>87</sup> Moreover, hypercholesterolemia is particularly associated with vulnerable plaque rupture, whereas acute thrombosis develops in smokers even without plaque rupture.<sup>76</sup> It would therefore appear that, in contrast to smoking, hypercholesterolemia-associated plaque rupture is such a dramatic event that thrombosis occurs despite preserved local t-PA release.

### Hypertension

Marked impairment of desmopressin-induced t-PA release but not vasodilatation has been demonstrated in hypertensives,<sup>60</sup> perhaps suggesting that impaired t-PA release may be a more sensitive marker of endothelial damage in hypertension. However, in contrast to the impaired desmopressin response, methacholine-induced t-PA release is unaffected.<sup>60</sup> Similar agonist-specific defects have been reported in smokers, in whom bradykinin<sup>62</sup> and substance P,<sup>53,83</sup> but not methacholine,<sup>62</sup> induced t-PA release is impaired. Although the explanation for this discrepancy is unclear, it may simply be more difficult to detect differences in release with a relatively weak stimulus, such as methacholine. Alternatively, hypertension and smoking may have different effects

on the specific pathways of secretion for each agent. However, the possibility that methacholine may be generally less able than other agents at discriminating meaningful differences in acute t-PA release between patient groups should be considered for future studies.

### Bradykinin and ACE Inhibition

Bradykinin is a potent endothelium-dependent vasodilator that has a brief duration of action caused by its rapid degradation by ACE.<sup>88</sup> In addition to being an inflammatory mediator, it is closely involved in the fibrinolytic and coagulation cascades. During the contact phase of blood coagulation, it is released after the cleavage of high-molecular-weight kininogen by kallikrein<sup>89</sup> and is a potent stimulant for endothelial t-PA release.<sup>20,32,40</sup> Thus, when plaque rupture or erosion activates the intrinsic coagulation pathway, liberation of bradykinin may provide important negative feedback to limit thrombus development. In keeping with this, bradykinin generation is transiently increased after an episode of unstable angina.<sup>90</sup>

Potentiation of endothelial t-PA release through the preservation of endogenous bradykinin might account for some of the cardiovascular benefits of ACE inhibitors. In support of this, in the forearm of healthy men<sup>91</sup> and patients with heart failure,<sup>28,92</sup> ACE inhibition augments bradykinin, but not substance P, induced t-PA release. Similarly, augmentation of bradykinin-induced t-PA release by ACE inhibition has been demonstrated in the coronary circulation.<sup>51,52</sup> The effect of ACE inhibition on t-PA release is mediated specifically through bradykinin, because angiotensin II receptor blockade has no effect on bradykinin-induced t-PA release.<sup>51,91</sup>

Bradykinin antagonism reduces local t-PA release during intra-brachial enalaprilat infusion,<sup>93</sup> providing direct evidence that ACE inhibition augments endothelial t-PA release through increased *endogenous* bradykinin. ACE inhibition might also enhance endothelial t-PA release by increasing bradykinin receptor expression.<sup>94</sup>

### Endothelial t-PA Release as a Novel Measure of Endothelial Function

The endothelium has a number of important functions, including regulation of vascular tone, coagulation, fibrinolysis, and inflammation. However, to date, most clinical studies on endothelial function have focused on endothelium-dependent vasomotion. Although this is a useful surrogate marker for the role of the endothelium in atherothrombosis,<sup>2</sup> measuring other aspects of endothelial function, including the capacity for t-PA release, may provide additional and novel insights.

Reports of preserved endothelium-dependent vasodilatation in smokers<sup>62</sup> and in patients with hypertension<sup>60,95</sup> despite reduced acute t-PA release suggest that, in some circumstances, reduced t-PA release may be a more sensitive marker of endothelial dysfunction. Moreover, some conditions associated with impaired endothelium-dependent vasodilatation, such as hypercholesterolemia, are not accompanied by reduced t-PA release.<sup>49</sup> These data highlight the complexity of vascular biology, and that it is perhaps naive to expect endothelial dysfunction to be expressed by a uniform

phenotype irrespective of the insult. Vascular inflammation is associated with augmentation of t-PA release despite impairing endothelium-dependent vasodilatation.<sup>73</sup> This calls into question the use of the term *endothelial dysfunction*. Is the cell truly dysfunctional or in an altered state of activation? In particular, the dominant use of this term to refer to endothelium-dependent and NO-dependent vasodilatation seems unnecessarily restrictive and inaccurate. The many and varied actions of the endothelium may be potentiated and inhibited simultaneously within the same cell. Whether this is ultimately beneficial or detrimental depends on the local and systemic setting of the individual vessel, tissue, and patient.

### Limitations of Clinical In Vivo Models

To date, most studies of dynamic endothelial t-PA release have been relatively small because of the invasive nature of the techniques. Measurement of coronary t-PA release is likely to be of greatest relevance to coronary pathophysiology but can only be performed in selected subjects undergoing coronary angiography. Whereas forearm assessment can be performed more widely, this vascular bed is less susceptible to atherosclerosis and subsequent thrombosis, raising questions on its validity as a surrogate for the coronary circulation. Nevertheless, consistent findings between the peripheral<sup>28,83,91</sup> and coronary circulations<sup>52,53</sup> support the notion that the forearm model is a reasonable surrogate.

Although quantitative assessment of endothelial t-PA release can be made using local infusions, the clinical models used are relatively simplistic when compared with the complex in vivo response to developing thrombus. For example, measuring t-PA release in response to components of the coagulation cascade, such as activated factor X or thrombin, might more closely mirror in vivo pathophysiology but would risk inducing arterial thrombosis. The in vivo clinical models also measure t-PA release across an entire vascular bed and it does not necessarily follow that such measures reflect the local capacity for t-PA release at the site of an eroded or ruptured plaque.

### Future Work

It can be reasonably hypothesized that reduced dynamic endothelial t-PA release predisposes to events such as myocardial infarction and unstable angina. However, longitudinal studies of t-PA release as a predictor of coronary events have not been performed. Whereas assessment of t-PA release is unlikely to become part of cardiovascular risk assessment, demonstrating that impaired release predicts cardiovascular events would support the hypothesis that reduced t-PA release is of pathophysiological significance.

Although the capacity for endothelial t-PA release is clearly reduced in some circumstances, the mechanisms are unknown. Serum from smokers, but not from nonsmokers, reduced substance P-induced t-PA release from cultured endothelial cells,<sup>96</sup> suggesting that a blood-borne mediator may affect endothelial t-PA responses. Using ex vivo human umbilical veins, increased intraluminal pressure decreased t-PA release as well as gene and protein expression,<sup>97</sup> suggesting that raised intraluminal pressure might itself impair t-PA release. Detailed characterization of the path-

ways of t-PA secretion and how these are affected by different stimuli will provide a platform for the investigation of the mechanisms of impaired secretion.

Improving t-PA release may represent a new therapeutic target in cardiovascular prevention. There is mounting evidence that some of the benefits of ACE inhibitors may be the result of enhanced t-PA release, whereas statins exert their beneficial effects through alternative pathways. Once the mechanisms of impaired t-PA release have been defined further, there is the potential to develop agents that will specifically augment endothelial t-PA release.

## Conclusions

The dynamic regulation of intravascular thrombus formation is central to our understanding of acute and chronic atherosclerotic events. The initiation, modulation, and resolution of thrombus associated with eroded or unstable coronary plaques are critically dependent on the efficacy of endogenous fibrinolysis. It is perhaps remarkable that this fundamentally important aspect of endothelial function has been relatively underinvestigated in clinical studies to date. Here, we have described the various methods of assessing acute endothelial t-PA release in humans and have explored the effects of various cardiovascular conditions and risk factors on the endogenous fibrinolytic capacity. Impaired endothelial t-PA release appears to be a particular feature of cigarette smoking and atherosclerosis but not hypercholesterolemia. Thus, endothelial dysfunction can be manifest in separate distinct pathways depending on the nature of the insult, underscoring the importance of critically investigating the specific consequences of vascular injury. The investigation of endothelial fibrinolytic function has the potential to provide major new insights into the pathophysiology of cardiovascular disease and to shape future therapeutic interventions.

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**PUBLICATION 37**

## Emerging Thrombotic Effects of Drug Eluting Stents

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**D**rug eluting stents (DES) have been enthusiastically introduced into contemporary interventional cardiology practice. By delivering locally active doses of antiproliferative drugs, they have demonstrated an impressive capacity to inhibit restenosis caused by neointimal hyperplasia; a complication that has plagued uncoated "bare-metal" stents (BMS). DES coated with either paclitaxel or rapamycin (sirolimus) are now deployed in over 90% of coronary interventions in the United States.<sup>1</sup> However, clinical follow-up has revealed significant concerns relating to the incidence of late (> 30 days) stent thrombosis, especially in long stents and after discontinuation of dual antiplatelet therapy.<sup>2</sup>

### See page 400

Stent thrombosis with DES has been attributed to various potential mechanisms (Figure), but delayed or incomplete stent endothelialization has been proposed as a major mechanism. In a recent autopsy study,<sup>3</sup> DES showed reduced endothelialization ( $27 \pm 26\%$  versus  $66 \pm 25\%$ ), especially in those with evidence of stent thrombosis. However, patients with DES had stents that were almost twice as long and included those receiving DES for indications not approved by the Food and Drug Administration, including acute myocardial infarction. This selected autopsy group represents the extreme end of the spectrum and may not reflect the situation in the majority of patients who receive DES and survive.

The hypothesis that late stent thrombosis is attributable solely to delayed endothelialization contrasts with porcine models of coronary stent implantation. Although endothelialization may be more rapid than in humans, endothelial coverage of DES and BMS in a porcine model is almost complete after 28 days with no significant difference between stents.<sup>4</sup> Furthermore, the bulk of evidence now suggests that reendothelialization is achieved by blood-borne endothelial progenitor cells originating from the bone marrow.<sup>5</sup> It is, therefore, hard to reconcile the hypothesis that locally active doses of antiproliferative agents impair reendothelialization by inhibiting the proliferation of adjacent endothelial cells.

With these observations in mind, Muldowney and colleagues in this issue of *Arteriosclerosis, Thrombosis, and Vascular Biology*, present a fascinating alternative and suggest a potential pathophysiological mechanism to explain the

occurrence of late stent thrombosis in DES.<sup>6</sup> Using microarrays of more than 11 500 genes, they report that, after incubation with either paclitaxel or rapamycin, the most consistent change in human endothelial gene expression was upregulation of the transcript for plasminogen activator inhibitor type 1 (PAI-1). They confirmed that these transcriptional effects were associated with increased PAI-1 antigen production. The upregulation of this antifibrinolytic factor presents an important potential mechanism underlying the prothrombotic effect of DES.

The fibrinolytic pathway describes a complex process involving the hydrolytic cleavage of fibrin, by plasmin, to cause clot dissolution. This process is regulated by a balance between the acute endothelial release of tissue plasminogen activator (t-PA) and its serpin inhibitor, PAI-1. Indeed, some have suggested that coronary thrombosis is particularly sensitive to imbalances in this fibrinolytic pathway.<sup>7</sup> Animal models of PAI-1 overexpression exhibit spontaneous macrovascular coronary thrombosis and myocardial infarction.<sup>8</sup> Plasma PAI-1 concentrations are independently predictive of adverse cardiovascular events in healthy populations<sup>9</sup> and patients with coronary heart disease.<sup>10</sup> Indeed, increased plasma PAI-1 concentrations are independently associated with major adverse cardiac events following BMS insertion,<sup>11</sup> and autopsy observations have further indicated that persistent fibrin deposition is a risk factor for late stent thrombosis with DES.<sup>3</sup>

Of further interest, Muldowney reports contrasting effects of paclitaxel and rapamycin on the expression and secretion of t-PA. Although rapamycin caused downregulation of t-PA mRNA expression and secretion, this effect was not seen in cells incubated with paclitaxel. Indeed, paclitaxel caused an increase in both expression and secretion in human umbilical vein endothelial cells. In the presence of impaired t-PA secretion, the prothrombotic effect of enhanced PAI-1 synthesis would be further amplified. Although other factors are likely to play a role, it is tempting to speculate that the combination of elevated PAI-1 and impaired t-PA secretion is a plausible explanation for the higher incidence of late stent thrombosis reported in one meta-analysis of patients treated with rapamycin-coated (Cypher, Cordis Corp) stents compared with paclitaxel-coated (Taxus, Boston Scientific Corp) stents.<sup>12</sup>

Muldowney and colleagues extended their preliminary in vitro findings by examining the effects of rapamycin and paclitaxel delivered to transgenic mice expressing enhanced green fluorescent protein (eGFP) linked to the human PAI-1 promoter. After 2 weeks of intraperitoneal infusion, the expression of eGFP-linked PAI-1 promoter was increased in coronary arteries, aorta, and kidney from animals treated with these antiproliferative agents. Thus they were able to confirm the consistency of their in vitro findings using an in vivo

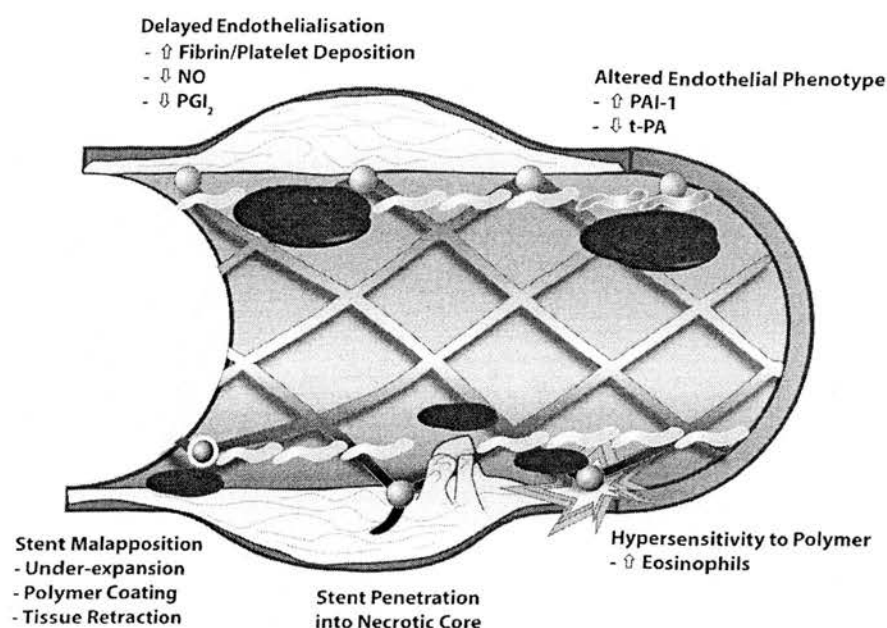
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Proposed mechanisms of drug eluting stent thrombosis.

model. Given that local PAI-1 expression is induced by percutaneous coronary intervention, the effects of rapamycin and paclitaxel would be anticipated to be enhanced even further at the site of stent implantation.

The dramatic reduction of in-stent restenosis associated with DES is an important therapeutic advance that has had a major impact on interventional cardiology. The small but significant absolute increase in late stent thrombosis is an important observation that remains a concern. Emerging mechanisms of DES thrombosis, including the induction of antifibrinolytic endothelial phenotypes, are helping us to understand this phenomenon. Such work will help inform the further refinement of this major therapeutic advance to allow physicians to have an effective and safe therapy for patients with obstructive atherosclerotic disease.

## Disclosures

None.

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